Activation of ATR-Chk1 Pathway Facilitates EBV-Mediated Transformation of Primary Tonsillar B-Cells

Dissertation
zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)
vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich
von

Vanessa Mordasini
aus
Muralto TI

Promotionskomitee
Prof. Dr. David Nadal (Vorsitz und Leitung der Dissertation)
Prof. Dr. Anne Müller
Prof. Dr. Cornel Fraefel
Prof. Dr. Christian Münz
Prof. Dr. Alessandro Sartori
PD Dr. Michele Bernasconi

Zürich, 2017

The experimental work presented in this thesis was performed at the division of infectious diseases at the University Children's Hospital Zurich. The supervision of this thesis was conducted by Prof. Dr. David Nadal (University Children's Hospital Zurich), Prof Dr Cornel Fraefel (Institute of Virology, University of Zurich), Prof Dr. Anne Müller (Institute of Molecular Cancer Research, University of Zurich), Prof Dr. Christian Münz (Institute of Experimental Immunology, University of Zurich), Prof Dr. Alessandro Sartori (Institute of Molecular Cancer Research, University of Zurich), PD Dr. Michele Bernasconi (University Children's Hospital Zurich). Mordasini *et al.*, Activation of atr-chk1 pathway facilitates ebv-mediated transformation of primary tonsillar b-cells, Oncotarget, 2016, Figure 1. created by author from data produced by Dr.med. Seigo Ueda.

The only aids used for composing this dissertation are those stated therein.

Zürich, 2017

Vanessa Mordasini

Table of contents

Summary

Epstein-Barr virus (EBV) is a member of the human gamma herpes virus family that benignly infects more than 95% of the population for life. In the infected host, EBV latently resides in a relatively stable pool of resting memory B cells. EBV infection is usually asymptomatic. Under specific circumstances, however, EBV seems to cause B-cell neoplasia including Burkitt's lymphoma, Hodgkin's disease, and post-transplant lymphoproliferative disorder (PTLD). Usually, proliferation of infected B cells is mainly controlled by a specific cytotoxic CD8+ T cell response. However, in immunocompromised patients this is no longer possible. Despite that, only a very small minority of EBV-infected humans develops EBV-associated malignancies, suggesting that mechanisms protecting from malignant transformation may be involved. The protective mechanisms are likely to be of host origin and may be usurped by EBV to insure survival of the host and thereby the own survival.

In the work presented here, we used primary B cells derived from tonsils, the portal of entry of EBV, to investigate how the DNA damage response might influence primary EBV infections during the first 96h. We identified for the first time that the EBV infection of tonsillar B cells induces a period of hyperproliferation at 72-96 hours after infection characterized by the activation of ataxia telangiectasia and Rad3-releated (ATR) and checkpoint kinase-1 (Chk1). Whereas inhibition of Chk1 did not affect B-cell transformation, the specific inhibition of ATR robustly decreased the transformation efficiency of EBV, rather than protect the cells against transformation.

In summary, our results suggest that the development of lymphoma following primary EBV infection in immunocompromised patients might depend from the activation of the specific ATR/Chk1 signaling pathway in infected B cells. In particular, activation of ATR seems to be key for EBV-induced B-cell transformation. Thus, targeting the interaction between ATR/Chk1 and EBV could offer new therapeutic options for the treatment of EBV-associated malignancies.

Zusammenfassung

Das Epstein-Barr Virus (EBV) gehört zur Familie der humanen gamma Herpes-Viren und führt bei rund 95% der Bevölkerung zu einer asymptomatischen lebenslangen Infektion. Im infizierten Wirt befindet sich das EBV in einem relativ stabilen Reservoir aus Gedächtnis B-Zellen. Unter speziellen Umständen scheint das EBV jedoch B-Zell Neoplasien wie das Burkitt-Lymphom, den Morbus Hodgkin oder Posttransplant lymphoproliferative Erkrankungen (PTLD) hervorzurufen. Normalerweise wird die Proliferation von EBV-infizierten B-Zellen durch eine spezifische zytotoxische CD8+ T-Zell Antwort kontrolliert. Bei immunsupprimierten Patienten ist dies jedoch nicht mehr möglich. Trotzdem kommt es nur bei einer Minderheit der infizierten Personen zu EBV-assoziierten Malignomen. Dies legt nahe, dass vor einer malignen Transformation schützende Mechanismen bestehen. Die protektiven Mechanismen gehen ursprünglich wahrscheinlich vom Wirt aus. Es ist jedoch möglich, dass das EBV sich diese angeeignet hat, um sein eigenes Überleben und auch jenes des Wirtes zu sichern.

In dieser Arbeit haben wir B-Zellen von Tonsillen, der Eintrittspforte des EBV, benutzt, um die Antwort auf die DNA Beschädigung in den ersten 96 Stunden einer EBV-Primärinfektion zu untersuchen. Wir haben erstmals gezeigt, dass die EBV-Infektion von tonsillären B-Zellen in den ersten 72-96 Stunden zu einer Hyperproliferation führt. Diese ist gekennzeichnet durch eine Aktivierung von ataxia telangiectasia, Rad3-releated (ATR) und checkpoint kinase-1 (Chk1). Die Inhibition von Chk1 beeinflusste die B-Zell-Transformation nicht. Demgegenüber konnte durch die Inhibition von ATR die Transformationsfähigkeit von EBV vermindert werden. Die EBV-infizierten B-Zellen konnten jedoch nicht gegen die Transformation geschützt werden.

Zusammenfassend legen unsere Resultate nahe, dass die Entwicklung eines Lymphoms als Folge einer EBV-Primärinfektion bei immunkompromittierten Patienten von einer Aktivierung des spezifischen ATR/Chk1 Signalwegs in den infizierten B-Zellen abhängig ist. Vor allem die Aktivierung von ATR scheint der Schlüssel für die EBV-induzierte B-Zell-Transformation zu sein. Demzufolge könnte die Interaktion zwischen ATR/Chk1 und EBV ein Angriffspunkt für neue therapeutische Massnahmen zur Behandlung von EBV-assoziierten Malignomen sein.

Introduction

1) Epstein-Barr virus (EBV)

Epstein-Barr virus (EBV) was discovered in 1964 by Anthony Epstein, Bert Achong and Yvonne Barr [2]. Together with the Kaposi's sarcoma Herpesvirus (KSHV) EBV belongs to the human γ-Herpes virus subfamily. Its tropism is mainly restricted to B-lymphocytes and epithelial cells. As a member of the *herpesviridae* family, EBV is an enveloped virus with a linear double stranded 184kbp long DNA [3]. EBV is extremely well adapted to man, in fact, more than 95% of the adult human population is benignly infected for life [4]. In the developing world, EBV primary infection usually occurs within the first 3 years of life and is mostly asymptomatic [3]. Nevertheless, if seroconversion is delayed to adolescence or young adulthood, like in western countries, primary EBV infection will increase the risk of developing acute infectious mononucleosis (IM) [3, 5]. IM is a self-limiting disease typically associated with sore throat, cervical lymphadenopathy, fever, and fatigue that usually resolve in a matter of month [6].

One common characteristic of herpesviruses is the ability to establish both a latent and a lytic infection. Latent and lytic EBV infections are characterized by expression of specific sets of genes and non-coding (nc) RNA, which can be activated to efficiently infect, persist and replicate within its host. EBV genes related to latency, together with ncRNA, are crucial to rapidly establish a pool of infected B-cell *in vivo* and for the transformation and proliferation of the infected B cells observed *in vitro* [7, 8]. Periodically, latent EBV reactivates and enters a lytic phase, which leads to the production of new viral particles that can infect new B cells and eventually spread to a new host via oral transmission [3, 6]. The lytic reactivation is initiated by the expression of the master lytic gene *BZLF1*, which encodes the immediate-early protein ZEBRA. ZEBRA is a transcription factor that initiates the lytic cycle cascade leading to expression of early and late lytic EBV gene [9]. *In vitro,* cell lines latently infected with EBV, e.g. the Burkitt's lymphoma (BL) derived Akata cell line, can be activated using diverse agents including phorbol esters (TPA), sodium butyrate (SB), transforming growth factor-β (TGF-β), or by B-cell receptor (BCR) cross-linking with anti-immunoglobulin G (anti-IgG) [10-14]. EBV can express up to nine different

latency genes: EBV nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and LP), latent membrane protein (LMP1 and LMP 2A-2B), according to four different types of latent gene expression programs (**Table 1**). Polymorphisms in the latent genes encoding the nuclear antigens EBNA2, -3A, -3B, and -3C determine two distinct types of EBV strains originally termed A and B and now designated as type 1 and 2. Type 1 and 2 can be distinguished by their prevalence among the population and their ability to transform B cells. Type 1 is the most abundant strain worldwide and the most efficient in transforming B cells, while type 2 is common in parts of Africa and is less efficient in transforming B cells. [15-17].

Type of infected B cells	Latency Program	Viral Genes	Function of the program
Naïve cells	Growth (Latency III)	EBNA1-6, EBNA-LP, LMP1, LMP2A-B, EBERs	Activate B cells
Germinal center cells	Default (Latency II)	EBNA1, LMP1, LMP2A, EBERs	Differentiate activated B cell into memory cells
Peripheral blood memory B cells	Latency (Latency 0)	EBERs	Allow life time persistence
Dividing peripheral blood memory B cells	EBNA-1 only (Latency I)	EBNA-1, EBERs	Replicate virus in plasma cells
Plasma cell	Lytic	All lytic genes	Replication of the virus

Table 1: Transcription programs used by EBV to maintain and establish infection (Adapted from [18])

Together with latency genes, virtually all latently infected cells express also a very high copy number of Epstein-Barr Virus-Encoded RNAs (EBERs), which have been suggested to play a role in the transformation of B cells and maintenance of EBV latency [19-23]. Following primary infection of the host through the oropharyngeal epithelium, EBV infects B cells inducing activation of the cell cycle. EBNA-LP and EBNA-2 are the first proteins to be expressed in the cells and allow expression of cellular and viral genes that will drive the transition from resting to proliferating B cells as well as promote activation of the pro-survival pathway (Latency III program) [24-26]. Although most of the proliferating cells will be eliminated by a specific immune-response, some will survive and originate the stable reservoir of EBV positive memory B cells. As memory B cells are long-lived resting B cells, EBV can completely switch off the expression of latent viral genes (Latency 0) protecting itself from the detection by the host innate immune-system [24, 27, 28]. EBV positive

memory B cells are subjected to the same physiological controls governing non-infected memory B cells. Thus, they can still occasionally divide, as a homeostatic mechanism to maintain a stable pool of memory B cells. For this reason, EBV can reactivate EBNA-1 expression (latency I) to ensure that the viral genome divides along with the cell [18, 29]. Furthermore, circulating memory B cells might encounter foreign antigens and home back to tonsils and be recruited into a germinal center reaction after which they may re-enter the pool or memory B cells or differentiate into plasma cells. Plasma cells, can home to mucosal sites in the oropharynx where the viral lytic cycle can be activated [30].

Albeit naïve and memory B cells *in vitro* seem to have the same susceptibility to EBV infection, with memory B cells being less efficiently transformed by the virus [31], the origin of the resting memory B cells pool is still a matter of debate. Two different hypotheses may explain the formation of the resting memory B cells pool. One hypothesis sees naïve B cells as the main target during primary EBV infection [30]. Proliferating EBV infected naïve B cells will then undergo germinal center reaction and become germinal center (GC) B cells, where EBV will express a more restricted set of genes (Latency II) [32]. Next, GC B cells will differentiate into memory B cells, the life-long reservoir of EBV. The second hypothesis envisages direct infection of preexisting memory B cells as a direct route to form the reservoir of EBV positive memory B cells [30].

1.1) Immune response against EBV

To protect ourselves against viral infection, toxins and other harmful substances we developed a variety of mechanisms to recognize, neutralize and in most cases eliminate the threat. The most important are the innate and the adaptive immune system. Although EBV can efficiently be recognized by the immune system during primary infection, the virus cannot be eliminated. It persists within the host, and establishes latent infection in memory B cells, establishing a restricted gene expression pattern that limits its recognition by the host immune system [3]. Nevertheless, each time the virus reactivates, its lytic cycle it will make it visible to the host's immune system, which will control and try to clear the infection by mean of a variety of immune effector mechanisms. The ability of EBV to persist lifelong in the host despite a powerful immune response indicates that the virus has developed several strategies to modulate, exploit and evade the immune system to ensure its on survival without arming the host.

The innate immune system is the first line of defence against pathogens and its activation is necessary to mount an efficient specific adaptive immune response. Activation of the innate immune system is initiated by the recognition of specific pathogens-associated molecular patterns (PAMPs) or danger associated molecular patterns (DAMPs), via specific pattern-recognition receptors (PRRs). PRRs include nucleotide-binding oligomerization domain (NOD) - like receptors (NLRs), retinoic acid-inducible gene I (RIG-I) - like helicase (RLHs) and Toll-like receptors (TLRs) which can be found in both innate and adaptive immune cells [33].

Toll-like receptors (TLRs) are one of the innate immune system's key factors implicated in the recognition of microbial structural motifs. TLRs family consists of ten different receptors able to recognize microbial-specific elements, including proteins, lipids, and nucleic acids derived from viruses, bacteria, parasites, and fungi [34]. B cells, the main EBV target, express TLRs 1, 2, 5, 6, 7, 9 [35]. Among these six receptors, the endosomal TLR9, which acts as a sensor for unmethylated CpG oligonucleotides (ODN) [36], has been shown to play an important role in the context of EBV infection [37]. TLR9 signaling pathway implies activation of a huge amount of proteins. Upon recognition of CpG ODN by TLR9, toll/interleukin-1 receptor (TIR) domain forms a complex with the TIR domain-containing adaptor myeloid differentiation primary response gene 88 (MyD88). Next, MyD88 recruits the interleukin-1 receptor-associated kinase (IRAK) 4, which in turn activates IRAK1. The latter associates with the TNF receptor associated factor (TRAF) leading to activation of mitogen-activated protein (MAP) kinases (JNK, p38, MAPK) and of the nuclear factor kappa B (NF-κB), which can promote cellular activation, proliferation and production of pro-inflammatory cytokines [38] We showed in our lab that triggering of the endosomal TLR9 with a synthetic unmethylated CpG DNA sequence, which mimic unmethylated EBV DNA, can block the reactivation of EBV lytic cycle induced by BCR cross-linking in BL derived Akata cells [39]. However, the components of the TLR9 signaling pathway that specifically interact and block EBV reactivation are still unknown.

1.2) EBV associated malignancies

EBV was originally discovered in B cell lines derived from Burkitt's lymphoma (BL) biopsies from Uganda [2]. EBV is the first human virus to be described as potentially tumorigenic and since is discovery, EBV infection has been implicated in the etiology of several B-cell malignancies like Hodgkin lymphoma (HL), human

immunodeficiency virus (HIV)-associated lymphoproliferative disorder, as well as post-transplant lymphoproliferative disorder (PTLD) (Table 2) [40-44]. Moreover, the discovery in the late 60's that EBV has the ability to infect and eventually transform B cells, resulting in the outgrowth of lymphoblastoid cell lines *in vitro,* reflects EBV's oncogenic potential [45-48]. In addition to B cells, EBV has the ability to infect T and natural killers (NK) cells, epithelial cells as well as smooth muscle cells in heavily immune-suppressed patients (Table 2) [3, 30, 44]. Infection of T and NK cells is very rare *in vivo* but it leads to a high risk of developing T and NK cell lymphoma [49, 50]. Infection of epithelial cells *in vivo* is more common and can lead, in particular in certain areas of China and south-east Asia, to the development of nasophariyngeal carcinoma (NPCas), as well as to development of gastric carcinoma (GC), where EBV is present in approximately 10% of the cases [3, 30]. In addition to EBV type 1 and 2 related polymorphisms; distinctive restriction fragment polymorphisms (RFPs) have been linked to specific EBV-associated cancer. It has been shown that in Southeast Asia, where the incidence of NPC is high, EBV type 1 is characterized by the loss of BamH1 restriction site at the right end of the viral genome as well as by a polymorphism in the LMP1 gene. On the other hand, in the Mediterranean region, where the incidence of NPC is medium to low, EBV type 1 is characterized by different RFPs and is known as the MABA strain. Interestingly, none of these polymorphisms can be found in EBV-associated B cell lymphomas, showing the importance that a small mutation in the viral DNA might have in the development of EBV-related malignancies [17, 51].

Immune-status	Cells of origin	Tumors	Latency
Immune-competent	B cells	Burkitt's lymphoma	I
		Hodgkins disease	II
	Non B cells	Nasopharyngeal carcinoma	I/II
		T/NK cell lymphoma	I/II
		Gastric carcinoma	I/II
Immune-compromised	B cells	PTLD	III
	Non B cells	Smooth muscle sarcoma	Unknown

Table 2: EBV-associated malignancies and associated viral latency. PTLD = post-transplant lymphoproliferative disease (adapted from [44])

Even though the majority of the human population carries the virus, EBV-associated malignancies are relatively rare. Since EBV first observation, a lot has been understood about the role of the immune-system and the importance of EBV latent-viral life cycle in the development of cancer. Despite that, the mechanisms that determine if an EBV carrier develops a tumor or not, still remain unclear. EBV associated malignancies can develop in both immunocompetent and immunocompromised individuals. BL, HL, T and NK cell lymphoma and the two types of carcinomas, all usually arise in immunocompetent patients after a relatively long period of viral latency ranging from 1-3 years to more than 30 years after primary EBV infection [3]. Moreover, the development of those specific types of malignancies strongly relates to geographical, environmental and genetic cofactors that increase the probability of their development [48]. On the other hand, other EBV associated malignancies develop only in an immunocompromised context caused by an advanced stage of acquired immune deficiency syndrome (AIDS), primary immunodeficiency (X-linked lymphoproliferative disorder) or by immunosuppressive treatment [3, 44, 48]. Immunocompromised patients lacking a fully functional immune-response are at higher risk of developing EBV-related malignancies compared to immunoompetent individuals. In this context, the period between primary EBV infection and the development of tumors is much shorter, usually less than 3 years, reflecting that EBV is both necessary and sufficient for the development of these malignancies [3].

1.3) EBV and Post-Transplant Lymphoproliferative Disorder

PTLD were first described in 1969 and are now a well-known complication after solid organ and hematopoietic cells transplantation [52]. PTLD are relatively common in the context of solid organ transplants, where up to 10% of transplanted patients are affected [53]. The majority of the cases (approximately 85%) are of B cells origin [54, 55], the remaining 15% arises from T cells and in some rare cases also from NK cells [56, 57]. Despite the high heterogeneity of PTLD, the medical community agrees that EBV, together with transplant-related immune-suppression, plays a critical role in development of the disease [58, 59]. Indeed, over 80% of PTLD of B cells origin are associated with EBV [53].

Both adults and children can be affected by PTLD, but the incidence is significantly higher in pediatric transplant recipients. Indeed, PTLD are the most

common form of post-transplant malignancies in children, whereas in adults they are the second most common post-transplant malignancy after skin cancer [60, 61]. PTLD can develop at any time after transplant but the majority of lymphomas are usually diagnosed within the first year and more than 90% of the cases are positive for EBV, while the percentage of EBV positive lymphoma decreases drastically in late onset PTLD [62].

Several risk factors are associated with the development of PTLD. EBV seronegativity at the moment of the transplant is a major risk factor increasing the probability of lymphoma development. This might explain why children, especially under the age of 10, have a higher risk for PTLD development after organ transplant [63]. Indeed, they are most likely to be EBV negative and to develop a primary EBV infection after transplant and therefore to develop PTLD. The type of organ transplanted is also an indicator for the risk of PTLD development. In both adult and children heart and lung transplant represent a higher risk compared to kidney and liver transplant for PTLD development [53]. This might be explained by the intensity of immunosuppression, which is much stronger in the context of thoracic organ transplant [53, 63]. Indeed, early onset EBV positive PTLD can be treated by reducing the immunosuppression and allowing the immune-system to naturally fight and eliminate malignant infected cells, underscoring the importance of a deficient immune system in the development of EBV related PTLD. More in detail, in immunocompetent people, EBV infection induces a strong CD8+ T cell response able to keep the infection under control. The T cell response induced by the virus is directed against specific viral epitopes that allows the immune-system to specifically target and kill infected B cells avoiding their uncontrolled proliferation and subsequent development of B cell lymphomas. Patients with impaired T cell response, on the other hand, lose the ability to control EBV induced B cell proliferation and are therefore highly susceptible to the development of EBV associated lymphoma.

Overall, despite the undoubted role of primary EBV infection and of the lack of a functional cellular immune-response, the percentage of patients that develop PTLD after transplant remains relatively low. This suggests that additional mechanisms might play a role in the development of post-transplant associated lymphomagenesis. One hypothesis might be that EBV alone may not be sufficient and additional mutations or pathways activation are necessary to drive B-cell transformation. On the other hand, the host might be able to activate additional

immune-mechanisms to keep the proliferation of the infected B cells under control despite the lack of EBV specific CD8+ T cells. One such additional mechanism could be the type of activated DNA damage response (DDR) pathway since it has been identified as a very important tumor suppressor mechanism upon EBV infection [64].

2) DNA damage response (DDR)

Both endogenous and environmental agents constantly challenge the integrity of the genetic information by inducing DNA damage. Spontaneous endogenous lesions mostly result from the normal cellular metabolic activity. They can be caused by dNTP misincorporation during DNA replication, exchange between DNA bases caused by deamination, loss of DNA bases after DNA depurination, or modification of DNA bases by alkylation as well as by reactive oxygen species (ROS) [65]. On the other hand, environmental damages to the DNA can be caused by chemical (chemotherapeutic agents like Cisplatin or Etoposide), as well as physical (UV-radiation, ionizing radiation (IR)). It has been calculated that both endogenous and environmental stresses together can induce up to 10^5 DNA lesions per day, making a powerful DDR mechanism essential for the maintenance of an error free DNA [66]. To protect their genome from DNA damage, eukaryotic cells have developed several powerful mechanisms to sense and repair DNA damage, thereby preventing genomic instability and avoiding tumorigenesis. The most important is the passage through three distinct cell cycle checkpoints (G1/S, intra-S and G2/M) before entering mitosis [67]. Active checkpoints induce cell cycle arrest, allowing time for repairs such as homologous recombination (HR), an error-free repair mechanism using an homologous DNA template and active during S or G2 phase, or non-homologous end joining (NHEJ), an error-prone repair mechanism that can ligate together two DNA strands without using an homologous DNA template and is active throughout the cell cycle [65]. If the damage to the DNA is too important, senescence or apoptosis will be activated to prevent spreading of mutations among the newly synthesized DNA that will result in the establishment of erroneous genetic information.

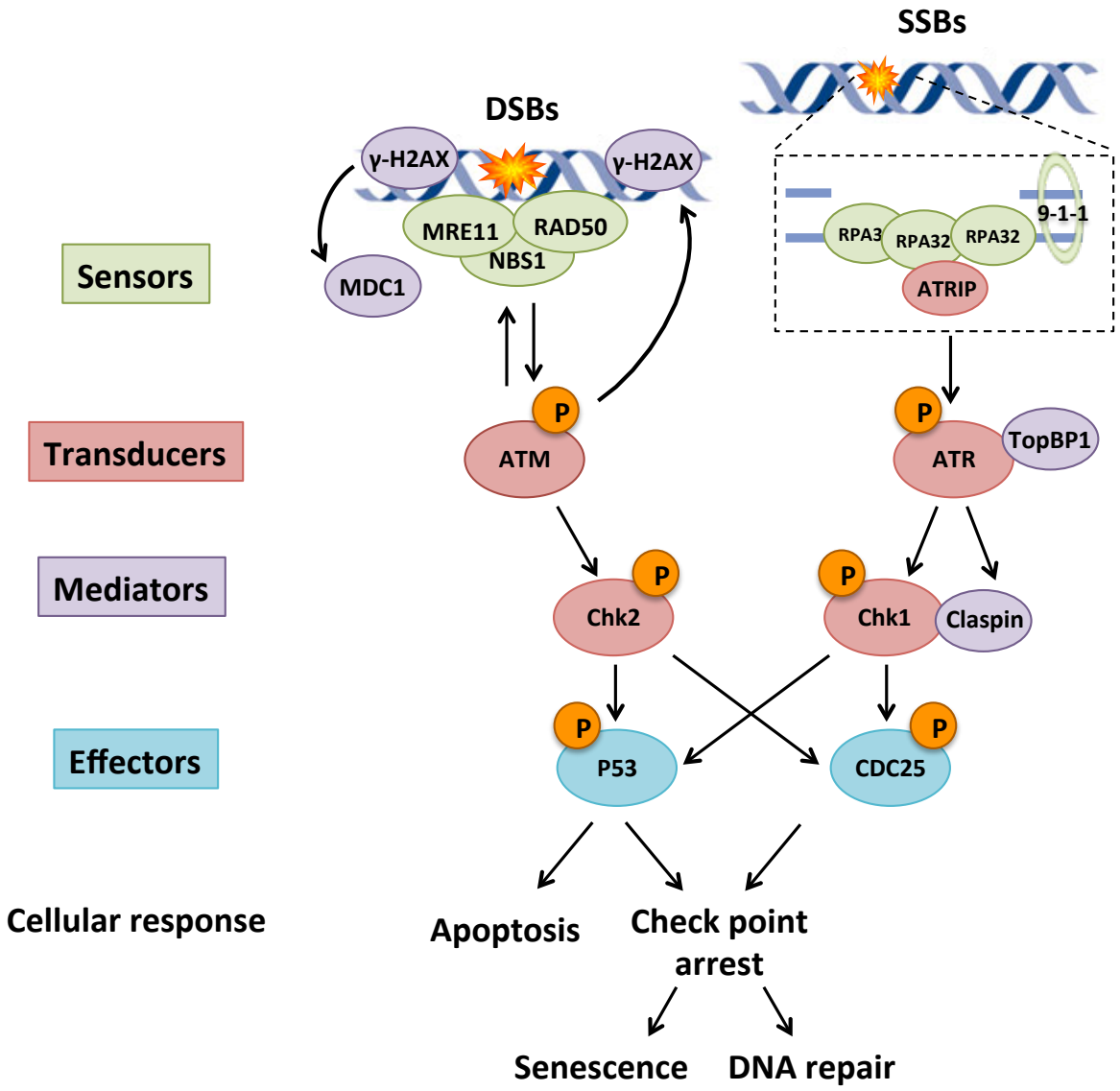


Figure 1: Activation of the ATM/Chk2 and ATR/Chk1 pathway. DSBs induce activation of ATM following recruitment of the MRN complex (MRE11-RAD50-NBS1) at the site of damage. Once activated, ATM modifies multiple substrates including γ-H2AX, MRN and the downstream effector kinase Chk2, leading to Chk2 activation and downstream signal transduction. SSBs induce activation of ATR following recruitment of RPA32, the 9-1-1 (RAD9-RAD1-HIS1) complex and the regulatory ATR interacting protein (ATRIP). Once activated ATR activates the downstream effector Chk1 with the support of TopBP1 and Claspin mediator proteins. The cellular response to ATM/Chk2 and ATR/Chk1 signalling is the activation of multiple DNA damage and replication checkpoint responses that can lead to DNA repair or cell death in case of irreparable DNA damage. Please refer to the text for further details and explanation.

2.1) Activation of the ATM/Chk2 and ATR/Chk1 DNA pathways

The nature of the DNA lesion that the cell might experience determines the specific DDR protein kinase that will be recruited to the site of DNA damage and thus, the specific DDR signaling pathway that will be activated (**Figure 1**). Activation

of DDR is mostly mediated by kinases of the phosphatidylinositol 3-kinase like protein family (PIKK3): ataxia telangiectasia and Rad3-related (ATR), ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinases (PK) [65].

DNA single-strand breaks (SSB) promote activation of ATR by the recruitment of the replication protein A (RPA), the regulatory ATR interacting protein (ATRIP) as well as the RAD9-RAD1-HIS1 (9-1-1) complex to the site of damage [68-70]. Accumulation of the latter will induce activation through phosphorylation of ATR, leading to the phosphorylation and activation of the downstream effector checkpoint kinase 1 (Chk1), the primary effector of the intra-S and G2-M checkpoints [71, 72]. Phosphorylation of Chk1 by ATR at serine (S)317 and S345 [73, 74] depends on the action of two mediator proteins: DNA-Topoisomerase-2 Binding Protein-1 (TopBP1) and Claspin. TopBP1 is recruited to the site of damage and is activated by the 9-1-1 complex and stimulates the activity of the protein kinase ATR [75, 76], whereas Claspin is activated by ATR and will bring Chk1 in close proximity to ATR to enable its phosphorylation [77, 78].

By contrast, DNA double-strand breaks (DSB) promote activation of ATM, through phosphorylation at S1981 [79] by the recruitment of the MRE11-RAD50-NBS1 (MRN) complex to sites of DSB [80, 81]. Once activated, ATM can phosphorylate many different downstream substrates such as the histone H2AX, the MRN complex itself and the downstream effector kinase checkpoint kinases 2 (Chk2). In order to amplify the local DDR across megabases flanking the site of damage, ATM phosphorylates the histone H2AX at S139 (γ-H2AX) [82]. The mediator of DNA damage checkpoint protein 1 (MDC1) will bind to γ-H2AX and recruit additional MRN and ATM amplifying the DDR, forming the so called γ-H2AX foci that can be detected by microscopy [83]. ATM phosphorylates the downstream Chk2 at threonine (T)68 [84]. Once activated, Chk2 dissociates from sites of damage and diffuses through the nucleus to activate multiple substrates mainly involved in the G1-S and G2-M checkpoints activation [71, 72, 85]. In addition, ATM has also been shown to phosphorylate the Krüppel-associated box (KRAB) – associated-protein-1 (KAP1) at S824 (pKAP1-S824) [86]. KAP1 has been described as a transcriptional repressor able to regulate transcription via changes in histone modifications at specific sites, to maintain the chromatin in a condensed state [87]. In response to DSB, ATM directly phosphorylates KAP1, which in turn will allow the heterochromatin to decondense at the damaged site, allowing the DDR machinery to reach and repair the DNA [86]. Since phosphorylation of pKAP1-S824 has been

shown to be maintained by the accumulation of active ATM at the site of damaged heterochromatin [88], it can be used as a marker for ATM activity at unrepaired site of DNA damage [89].

2.2) DDR and the activation of cell cycle checkpoints

As mentioned above, the ATR/Chk1 and ATM/Chk2 activation are strictly related to the cell cycle regulation and to activation of cell cycle check points. The activity of these kinases halts the progression of the cell cycle, activating and supporting various repair mechanisms, and promoting programmed cell death and senescence when the genome is excessively damaged. Which checkpoint will be activated is determined by Chk-mediated phosphorylation of Cdc25 phosphatases and p53 that can influence cell cycle progression and apoptosis [90] (**Figure 2**). Activation of the ATM/Chk2 pathway activates the G1-S checkpoint through inhibition/degradation of the kinase Cdc25A and stabilization of p53. Both proteins are phosphorylated directly by Chk2, and are key factors necessary for the S phase transition. Shortly, phosphorylation of Cdc25A induces its degradation and blocks its phosphatase activity, which is necessary for the progression from G1 to S phase [91]. Phosphorylation of p53, on the other hand, activates and stabilizes the protein inducing the up regulation of the cyclin-dependent kinase inhibitor p21, which blocks entry in the S-phase [92]. The intra-S checkpoint, on the other hand, is regulated by the activation of the ATR/Chk1 pathway specialized in the recognition of SSB following occurrence of replication stress. Slowing or stalling in replication fork progression is what defines cellular replication stress, which results in the generation of single stranded DNA recognized by the ATR/Chk1 pathway [93]. Similarly to Chk2, activation of Chk1 blocks the activity of the phosphatase Cdc25A, in this case preventing firing of new replication origins, blocking DNA replication and therefore arresting the cell in S-phase to permit repair of the lesions [94]. The G2 checkpoint is essential to prevent division of cells with unresolved DNA lesions. To induce a sustained G2/M checkpoint arrest both Chk2 and Chk1 are necessary. DSB occurring in G2 induce activation of the ATM/Chk2 pathway and phosphorylation of the phosphatase Cdc25C by Chk2. Processing of these lesions creates SSB that are immediately recognized by the ATR/Chk1 pathway, which also phosphorylates Cdc25C leading to a rapid G2 arrest [71, 91, 95].

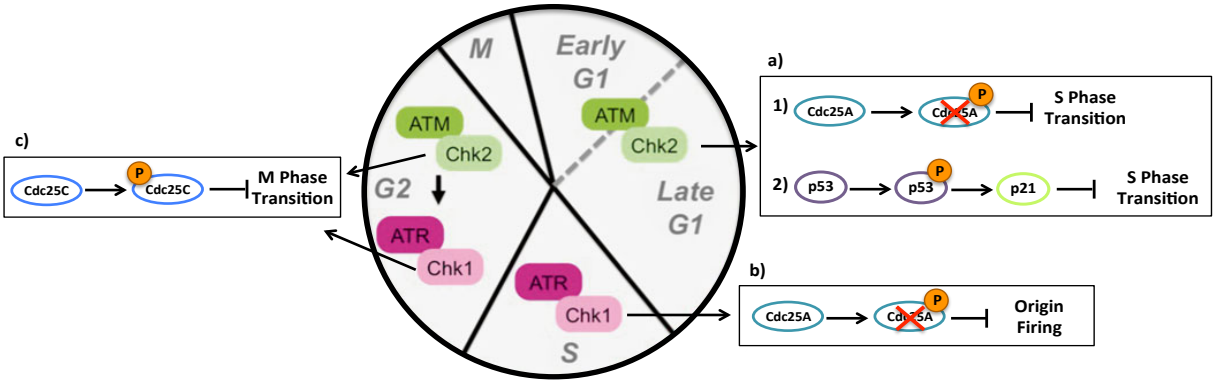


Figure 2: ATM/Chk2 and ATM/Chk1 cell cycle regulation. ATM and Chk2 are responsible for the activation of G1/S and G2/M checkpoints. ATR and Chk1 are activated by single stranded DNA damage and are therefore responsible for the activation of the intra-S checkpoint in response to stalled replication forks and/or DNA replication associated damages. a) Activation of G1/S checkpoint. 1) Phosphorylation of Cdc25A by Chk2 suppresses its phosphatase activity and induces its degradation blocking the progression into S-phase. 2) Phosphorylation and activation of p53 by Chk2 stabilizes the protein inducing upregulation of the cyclin-dependent kinase inhibitor p21 b) Activation of the intra S-phase checkpoint. Similarly to CHk2, activated Chk1 phosphorylates and blocks activity of Cdc25A inhibiting firing of replication and DNA replication, blocking the progression into G2 and mitosis. c) Activation of the G2/M checkpoint. In order to activate the G2/M checkpoint, both activated Chk2 and Chk1 need to phosphorylate the phosphatase Cdc25C in order to block progression into M-phase. (Adapted from [1])

3) Viral infection and DNA damage response activation.

Viruses are obligatory intracellular pathogens that strictly depend from the host cellular machinery for efficient replication. The great majority of processes that requires cooperation between the virus and the host cell machinery involve DNA replication. Therefore, viruses have evolved strategies to manipulate the host cell cycle to create a cellular environment favorable for viral DNA replication. It is therefore not surprising that the DDR machinery has been shown to play an important role during infection of the host.

Several DNA viruses have been shown to activate the cellular DNA damage response. Among these, we can find members of the *herpesviridae* family such as herpes simplex virus (HSV)-1 [96-98], HSV-2 [99], human herpes virus (HHV)-6 [100], Kaposi's sarcoma [101] EBV [102-104]; as well as other DNA viruses such as simian virus 40 (SV40) [105, 106], human papilloma virus (HPV) [107] and human cytomegalovirus (HCMV) [108].

Upon infection and replication, a large amount of exogenous viral DNA is present in the host cell and possible abnormal viral genome secondary structures are likely to

be recognized by the DDR machinery providing an antiviral cellular response protecting the host cells from the infection. Indeed, it has recently been shown by Saha *et al.* [109] that adenoviruses (Ads) infection induces an anti-viral local MRN-ATM-γH2AX response that restricts only replication of the viral genome without jeopardizing the cellular viability. Detection of foreign DNA prior to replication has also been observed upon HSV-1 infection. HSV-1 DNA enters the cells as linear dsDNA and is immediately recognized by the cellular DDR machinery, which will rapidly create a transcriptional inhibitory environment by deposits of repressive ubiquitin marks on the viral DNA [96]. Interestingly, triggering of the DDR is not restricted to DNA viruses infection. Retroviruses, which are RNA viruses depending on a DNA intermediate, have been shown to induce activation of the DDR especially during integration of the viral genome into the host DNA that require formation of DSB (reviewed in [110]).

Viruses have developed very elegant mechanisms to escape the antiviral response provided by the activation of the host DDR. For example, cellular DDR proteins are targeted for degradation by Ads [111-113] and HSV [114-116] viral ubiquitin ligases. Viral Ads and HSV proteins also have the ability to directly interact with cellular DDR proteins inducing mislocalization of the latter with subsequent shutting down of the DDR [117-119]. For some viruses like MHV68, KHSV and HCV the direct protein-protein interaction between viral and cellular components (in these three cases ATM) is sufficient to block the DDR allowing the virus to replicate in the host cell [120-122].

The DDR is not always detrimental for the virus, despite its antiviral properties. Viral genome replication takes place in complex molecular structures known as viral replication complexes (VRC) (reviewed in [123]) resulting from the association of the viral DNA and the hijacked cellular replication machinery. Together with these cellular replication components, cellular DDR factors including MRN, ATM, RPA, ATRIP, ATR and γH2AX are often recruited into VRC, and are required for a proper viral lytic replication [124]. HSV-1 [98, 99, 125], as well as EBV [126, 127] and HCMV [128], have been shown to recruit DDR factors into their own VRC. Albeit the precise role of cellular DDR factors in VRC still remain to be clearly understood, it has been suggested that DDR factors might play a role as viral DNA proof-reader before the viral genomic information is packed into new virions and released from the cell, as well as in the resolution of viral replication intermediates and/or expression of viral genes [124]. Despite the advantage for viral replication, activation of the DDR can

still trigger activation of signaling pathways leading to cell death through the activation of several downstream effectors such as p53. To prevent such an threatening side effect, viruses have developed strategies to target and block the activation of this type of proteins in order to avoid killing of the host cell and elimination of the virus [129].

3.1) EBV and the DNA damage response

EBV has been shown to interact with the host DDR in all phases of its lifecycle. Interestingly, depending from the viral life cycle stage, activation of the DDR can be either beneficial or detrimental for the persistence of the virus. In fact, the DDR machinery can either be hijacked by EBV and be used to facilitate the infection of the cells or can be activated by the host cell as an additional antiviral response to block EBV infection.

As described above, EBV infection of the host B cells induces proliferation as well as activation of survival pathways dramatically increasing the risk of malignant transformation. However, the development of EBV related malignancies is rare despite the high prevalence of seropositive people and the efficiency of EBV B cell transformation is very low (1% of immortalization after infection) [45, 130]. Therefore, the host must have developed a mechanism to limit the transformation efficiency of EBV. Nikitin and colleagues showed that activation of the ATM/Chk2 signaling pathway during the hyperproliferative phase, which follows *in vitro* EBV infection of peripheral blood B cells (PBMC), is critical for the suppression of EBV-mediated cell transformation and can act as an innate tumor suppression pathway [64]. This observation is supported by gene expression microarrays analysis indicating that hyperproliferating cells express more genes related to cell cycle progression and DDR compared to LCLs or uninfected B cells [64, 131, 132].

To counteract the effect of the DDR and establish latency, EBV must have developed a mechanism to block this host antiviral response. The latent protein EBNA3C has been shown to directly interact with DDR components such as Chk2, releasing the G2/M checkpoint arrest [133], or to inhibit the expression p16, which is important in the activation of senescence in case of irreparable DNA damage, via epigenetic regulation [134, 135]. Infection with an EBNA3C knockout virus increases the DDR and reduces the transformation efficiency, confirming these observations [64]. LMP1 has also been shown to down regulate ATM expression, decrease Chk2

phosphorylation and, as a consequence, to inactivate the G2 checkpoint [136]. From the host side, on the other hand, interfering with the cellular DDR might increase chromosomal instability and explain the development of EBV related malignancies.

On the other hand, EBV is able to usurp and use the cellular DDR machinery for its own benefit during both latent and lytic viral DNA replications. During its latent viral life cycle, EBV specifically activates Chk2 and recruits the MRN complex to the origin of the EBV plasmid replication (oriP) in order to successfully and faithfully replicate [137, 138]. In the absence of Chk2 and/or Nbs1 (component of the MRN complex) oriP replication and the maintenance of the episomal DNA are compromised, indicating an important role for Chk2 and MRN during latent replication [137, 138]. As describe above (Chapter 1: Epstein-Barr virus (EBV)), under specific conditions EBV can switch from a latent to a lytic life cycle. Switch to the lytic phase requires expression of the two immediate early lytic proteins ZEBRA and BRLF1 as well as the early protein BMRF1 (also known as early antigen protein D or EA-D). It has recently been shown by Wang'Ondu *et al.* [102] that ZEBRA is able to induce activation of ATM via recruitment of the chromatin remodeling protein TIP60, which has been shown to activate ATM in response to DDR after binding to its own promoter [139]. Together with ATM, MRN, γH2AX and p53 will also be recruited to the VRC and lead to the maximal autoactivation of the ZEBRA promoter increasing the level of ZEBRA and BMRF1 [102, 140] and leading to the full activation of EBV lytic replication. Together with ZEBRA and BMRF1, the early lytic protein BGLF4, a EBV serine/threonine kinase conserved among Herpesviruses, has also been shown to play a role in the activation of ATM by directly phosphorylating Tip60 and therefore reinforcing the effect of ZEBRA [102, 141]. Interestingly, despite the activation of the ATM checkpoint that blocks cells in G0 and G1, the signaling down-stream of p53 is blocked protecting the infected cell from apoptosis and allowing progression into an S-phase like environment providing access to the cellular replication machinery [129, 140, 142]. The induction of cell cycle arrest enables the virus to ensure that cellular DNA synthesis is blocked before the virus begin its own DNA replication, limiting competition for resources between the cellular and viral replication [143].

Subject of investigation

The aim of my thesis was to investigate the activation of the DNA damage response in tonsillar B cells (TBCs) and its effect on Epstein Barr virus (EBV) - induced B cell transformation. To address this subject in detail, the following topics were investigated:

1) **Does EBV primary infection induce activation of the DDR in tonsillar B cells?**

Primary EBV infection of peripheral B cells has been shown to activate ATM/Chk2 specific pathway shortly after infection reducing EBV transformation efficiency [64]. Preliminary results obtained in our lab showed that in EBV-infected TBCs a different DDR pathway seemed to be activated during primary EBV infection, compared to what has been shown by other groups [64, 144]. Here, we investigate if inoculation of tonsillar B cells, the portal of entry of EBV and therefore a more suitable in *vitro* model to study primary EBV infection, induces activation of the same DDR pathway.

2) **How does primary EBV infection activates DDR?**

Interaction between EBV and the cellular DDR has been deeply analyzed in past years. A substantial body of literature is available regarding the function of the DDR during latent and lytic EBV infection. On the other hand, very little information is available regarding activation of the DDR during primary EBV infection. Here, we try to understand the mechanism leading to the activation of a specific DDR pathway (ATR/Chk1pathway) during the first 96h post EBV inoculation *in vitro*.

3) **What is the impact of ATR/Chk1 activation on EBV dependent B cell transformation?**

EBV has the ability to induce growth transformation of B cells *in vitro*. In a healthy person infected by EBV, uncontrolled B cell proliferation is mainly controlled by the adaptive immune-system with a specific CD8+ T cell response. In

immunocompromised patients, however, this is no longer possible. It has been suggested by Nikitin *et al.* that the specific ATM/Chk2 DDR pathway can decrease EBV dependent transformation of peripheral blood B cells [64]. Here, we investigate the effect of ATR/Chk1 inhibition on EBV dependent TBCs transformation *in vitro*, using specific ATP competitive inhibitors for ATR and Chk1.

4) Does IRAK4 and TLR9 mutation in Akata cells affects BCR signaling?

We showed in our lab that Akata IRAK4 ko and TLR9 ko cells cannot be lytically reactivated by IgG cross-linking as efficiently as wild type Akata cells. BZLF1 mRNA level is strongly amplified in Akata cells upon BCR cross linking, whereas we observe a 3-8 fold lower expression in IRAK4 ko an 2 fold lower expression in TLR9 ko. Here, we investigated whether a decreased expression activation of downstream effectors molecules such as PI3K, MAPK, and say the rest might explain the reduced level of BZLF1 expression in IRAK4 CRISPR/Cas9 single cell clones.

Results

1) Activation of ATR-Chk1 pathway facilitates EBV-mediated transformation of primary tonsillar B-cells

Vanessa Mordasini, Seigo Ueda, Roberta Aslandogmus, Christoph Berger, Claudine Gysin, Daniela Hühn, Alessandro A. Sartori, Michele Bernasconi, and David Nadal

Manuscript published

Abstract

Primary infection of the immunocompromised host with the oncovirus Epstein-Barr virus (EBV) that targets mainly B-cells is associated with an increased risk for EBV-associated tumors. The early events subsequent to primary infection with potential for B-cell transformation are poorly studied. Here, we modeled in vitro the primary infection by using B-cells isolated from tonsils, the portal of entry of EBV, since species specificity of EBV hampers modeling in experimental animals. Increasing evidence indicates that the host DNA damage response (DDR) can influence and be influenced by EBV infection. Thus, we inoculated tonsillar B-cells (TBCs) with EBV-B95.8 and investigated cell proliferation and the DDR during the first 96 hours thereafter. We identified for the first time that EBV infection of TBCs induces a period of hyperproliferation 48-96 hours post infection characterized by the activation of ataxia telangiectasia and Rad3-releated (ATR) and checkpoint kinase-1 (Chk1). Whereas inhibition of Chk1 did not affect B-cell transformation, the specific inhibition of ATR robustly decreased the transformation efficiency of EBV. Our results suggest that activation of ATR is key for EBV-induced B-cell transformation. Thus, targeting the interaction between ATR/Chk1 and EBV could offer new options for the treatment of EBV-associated malignancies.

For detailed information, see attached manuscript 1

2) IRAK4 is essential for TLR9-induced suppression of Epstein-Barr virus *BZLF1* transcription in Akata Burkitt's lymphoma cells

Marc Jordi, Jeannine Marty, Vanessa Mordasini, Anna Lünemann, Scott McComb, Michele Bernasconi, David Nadal

Manuscript submitted for publication

Abstract

Burkitt's lymphoma (BL) is the most common cancer in children of equatorial Africa, and is endemic to areas where people are chronically co-infected with Epstein-Barr virus (EBV) and the malaria pathogen Plasmodium falciparum. Although the contribution of both pathogens in the oncogenic process remains poorly understood, we showed earlier that the activation of Toll-like receptor (TLR) 9 by hemozoin, a disposal product formed from the digestion of blood by P. falciparum, suppresses the lytic reactivation of EBV in BL cells. The ability of latent EBV to evade the immune system, and its unique oncogenic potential, might thus be reinforced by co-infection with P. falciparum. Here, we explore in the BL cell line Akata the mechanism involved in repression of the viral transcription factor BZLF1, the master gene controlling EBV lytic reactivation, mediated by TLR9. We show that BZLF1 repression is reinforced by TLR9 engagement via a mechanism that is largely independent of de novo protein synthesis. By CRISPR/Cas9-induced inactivation of TLR9, MyD88, IRAK4 and IRAK1 we confirm that this suppression is dependent on functional TLR9 and MyD88 signaling and identify IRAK4 to be essential for TLR9-induced suppression of BZLF1 expression upon BCR cross-linking. Our results unprecedentedly show that TLR9-mediated inhibition of lytic EBV is largely independent of new protein synthesis and demonstrate the central roles of MyD88 and IRAK4 in this process contributing to EBV's persistence in the host's B-cell pool.

For detailed information, see attached manuscript 2

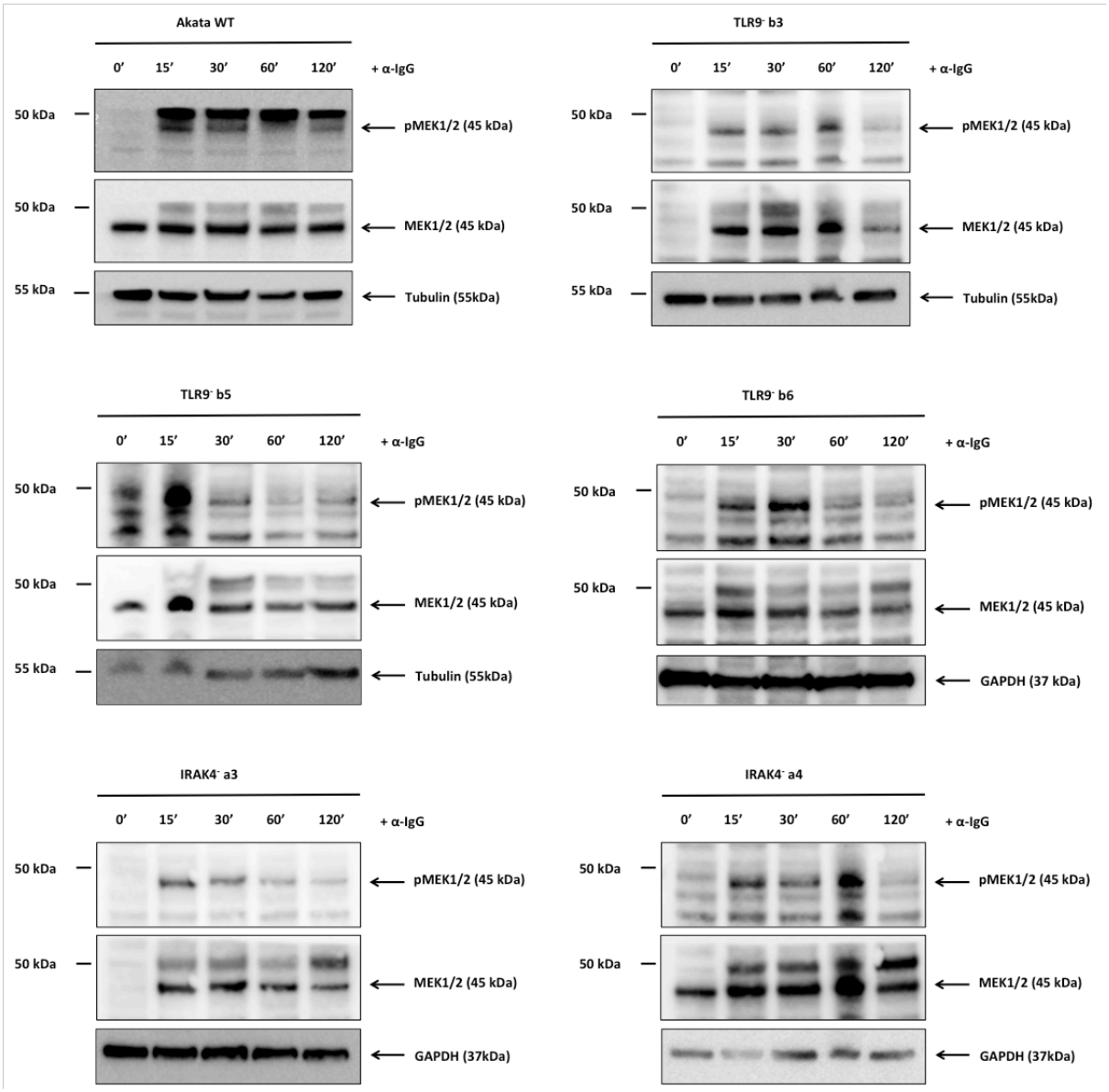
2.1) Materials and Methods

Cell lysis and Western blot. Total protein lysates were obtained after lysing 10^6 cells in RIPA complete buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 2mM EDTA, 1% NP40 complemented with 0.1% SDS, 1 x EDTA-free protease inhibitor cocktail (Roche, Rotkreuz, Switzerland)). Cell extracts were passed 10 times through a 25-G syringe. Protein content was determined using the Pierce BCA Protein Assay Kit (ThermoScientific, Zug, Switzerland), according to the manufacturer's instructions. To analyze protein expression by western blot, protein (20 mg/well) was loaded into a NuPAGE 4–12% Bis-Tris Gel (Life Technologies), subjected to SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare, Glattbrugg, Switzerland). The membrane was incubated with rabbit anti-Akt (#9272), rabbit anti-pAkt (#4058), rabbit anti-Mek1/2 (#9122), rabbit anti-pMek1/2 (#2338), rabbit anti-Syk (#12358), rabbit anti-pSyk (#2710) or rabbit anti-β-actin (#4967, all from Cell Signaling Technology, Allschwil, Switzerland) primary antibodies; subsequently with anti-rabbit (#7074) or anti-mouse (#7076) IgG HRP-linked secondary antibodies (Cell Signaling Technology). The signal was detected with the ECL Western Blotting Detection Reagents (GE Healthcare) and imaged using the LAS-3000 image reader (Fujifilm, Dielsdorf, Switzerland).

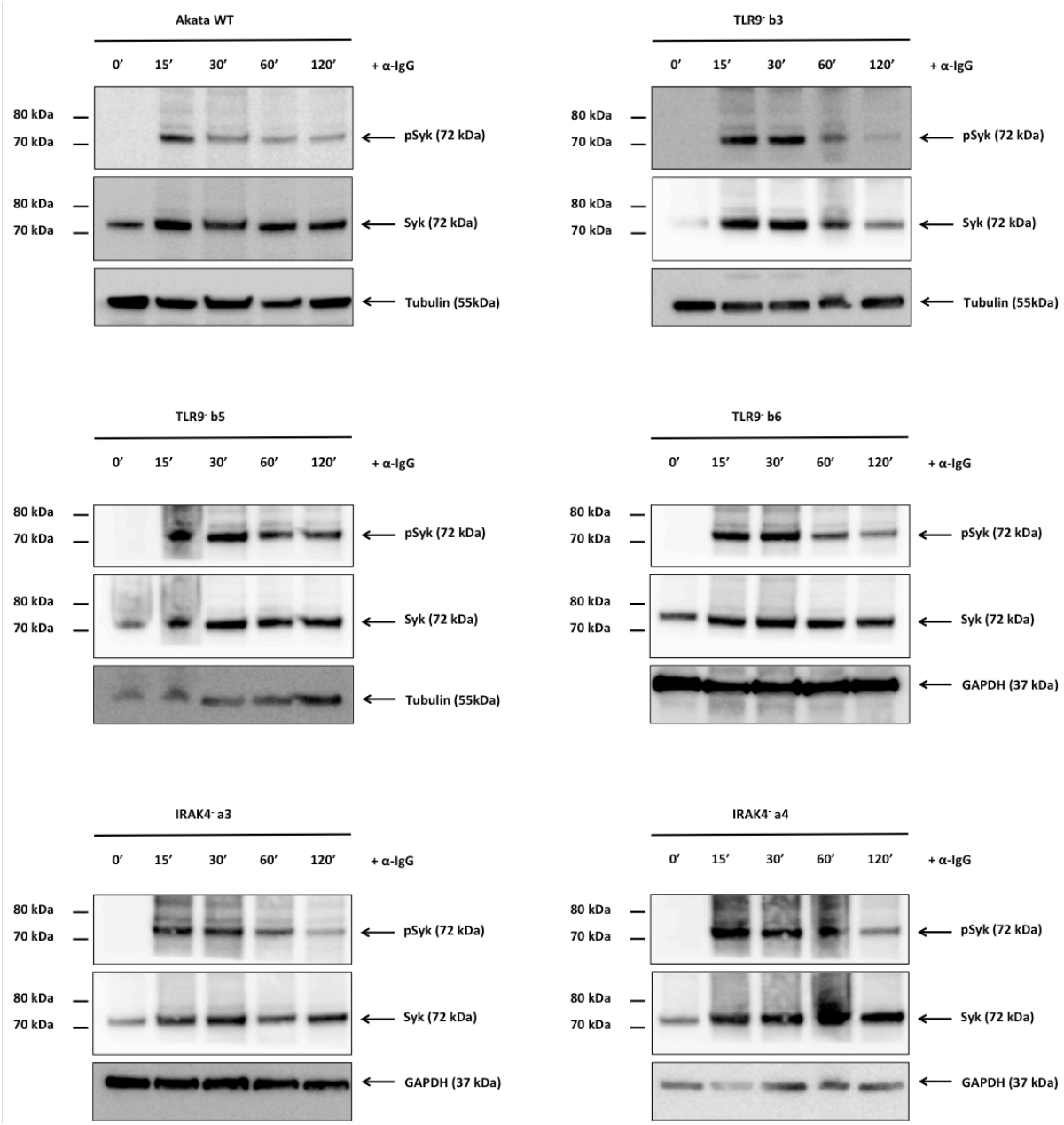
2.2) Results

Previous experiment performed in our lab showed that the IRAK4 and TLR9 mutated Akata cell line cannot be efficiently lytically reactivated by IgG cross-linking. Therefore, we wanted to investigate whether other in those two specific ko cell lines, markers downstream of BCR signalling (Mek, Syk and Akt) were down regulated compared to WT Akata cells. In order to investigate if the BCR signaling is affected by mutation of IRAK4 and TLR9, we stimulated the CRISPR/Cas9 single cell clones IRAK4- a3, IRAK4- a4, TLR9- b3, TLR9- b5 and TLR9- b6 with anti-IgG for 15, 30, 60 and 120 minutes. The phosphorylation levels of Mek, Syk and Akt were measures by western blot and compared with the levels measured in Akata WT cells. The results show an increased Mek, Syk and Akt phosphorylation upon treatment with anti-IgG with only minor differences between the IRAK4- and TLR9- clones and the Akata WT cells (**Figure 3** and **Figure 4**). Thus, we can exclude an influence of

IRAK4 or TLR9 abrogation on the BCR pathway to be the reason for the differences in BZLF1 mRNA expression upon lytic reactivation.



B


Akata WT
0’ 15’ 30’ 60’ 120’ + α-IgG
80 kDa
70 kDa
pSyk (72 kDa)
Syk (72 kDa)
Tubulin (55kDa)
TLR9- b3
GAPDH (37 kDa)
TLR9- b5
TLR9- b6
IRAK4- a3
IRAK4- a4

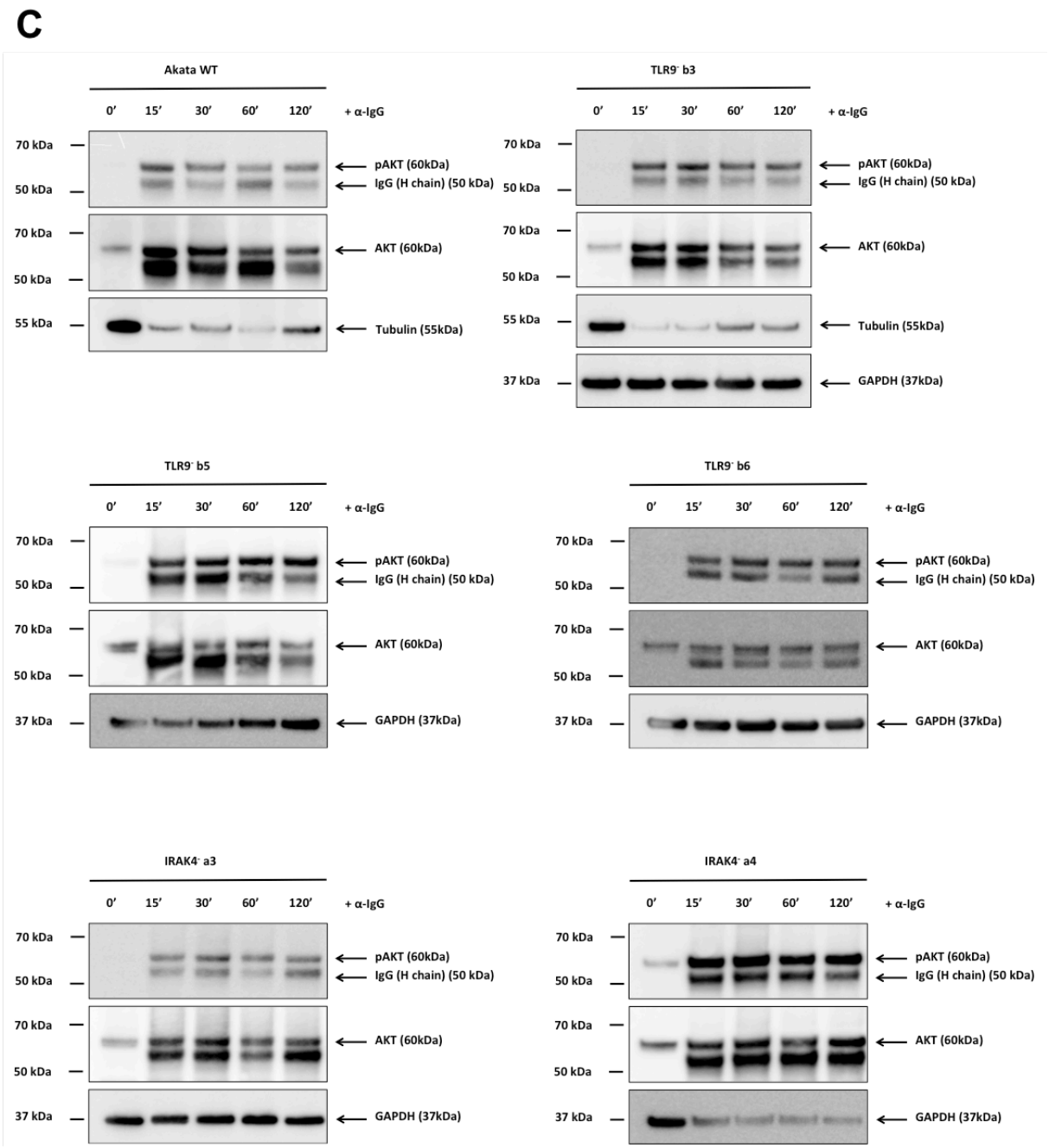
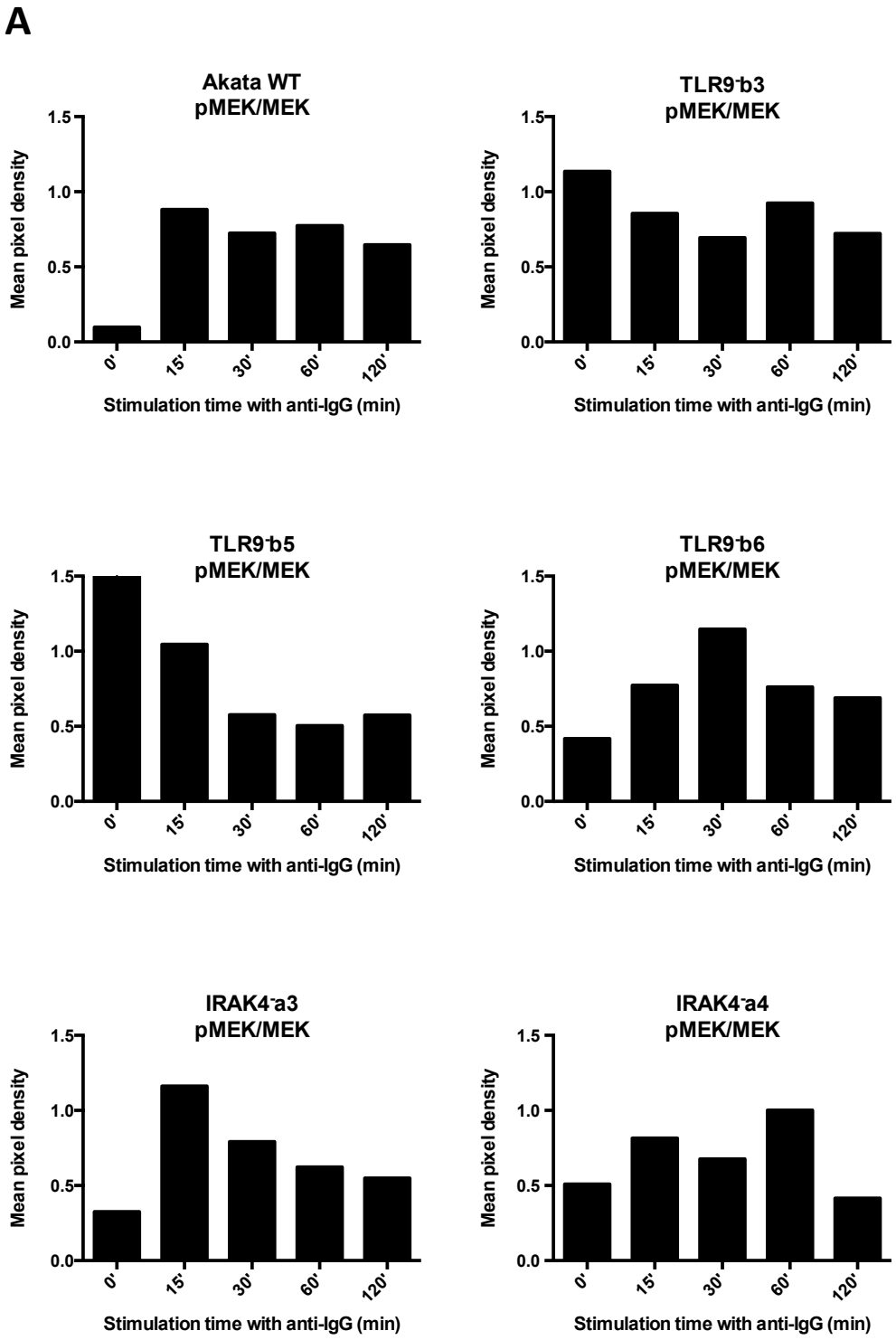
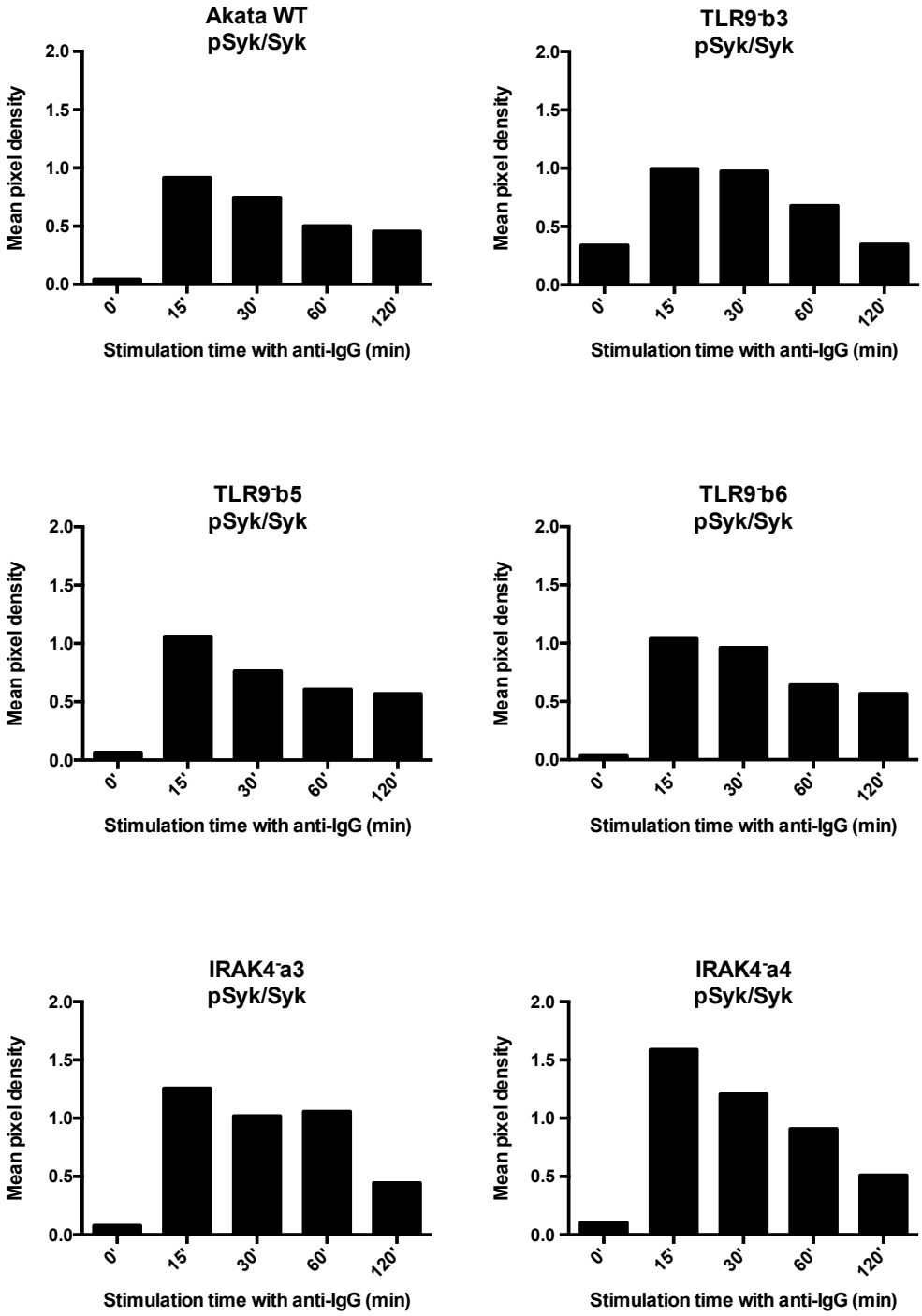


Figure 3: IRAK4 and TLR9 ko do not affect BCR signalling: Akata WT cells and TLR9 (TLR9-b3, TLR9-b5 and TLR9-b6) and IRAK4 (IRAK4-a3 and IRAK4-a4) CRISPR/Cas9 single cell clones were treated with ODN CpG 2006 for 15', 30', 60' and 120'. Cells were harvested at the indicated time points and expression of total (A) MEK and pMEK, (B) Syk and pSyk and (C) Akt and pAkt were analyzed by western blotting. Expressions of Tubulin or GAPDH were measured as loading control.


A
Akata WT
pMEK/MEK
TLR9-b3
pMEK/MEK
TLR9-b5
pMEK/MEK
TLR9-b6
pMEK/MEK
IRAK4-a3
pMEK/MEK
IRAK4-a4
pMEK/MEK
Mean pixel density
1.5
1.0
0.5
0.0
0'
15'
30'
60'
120'
Stimulation time with anti-IgG (min)

B


Akata WT
pSyk/Syk
TLR9-b3
pSyk/Syk
TLR9-b5
pSyk/Syk
TLR9-b6
pSyk/Syk
IRAK4-a3
pSyk/Syk
IRAK4-a4
pSyk/Syk
Mean pixel density
0.0
0.5
1.0
1.5
2.0
0'
15'
30'
60'
120'
Stimulation time with anti-IgG (min)

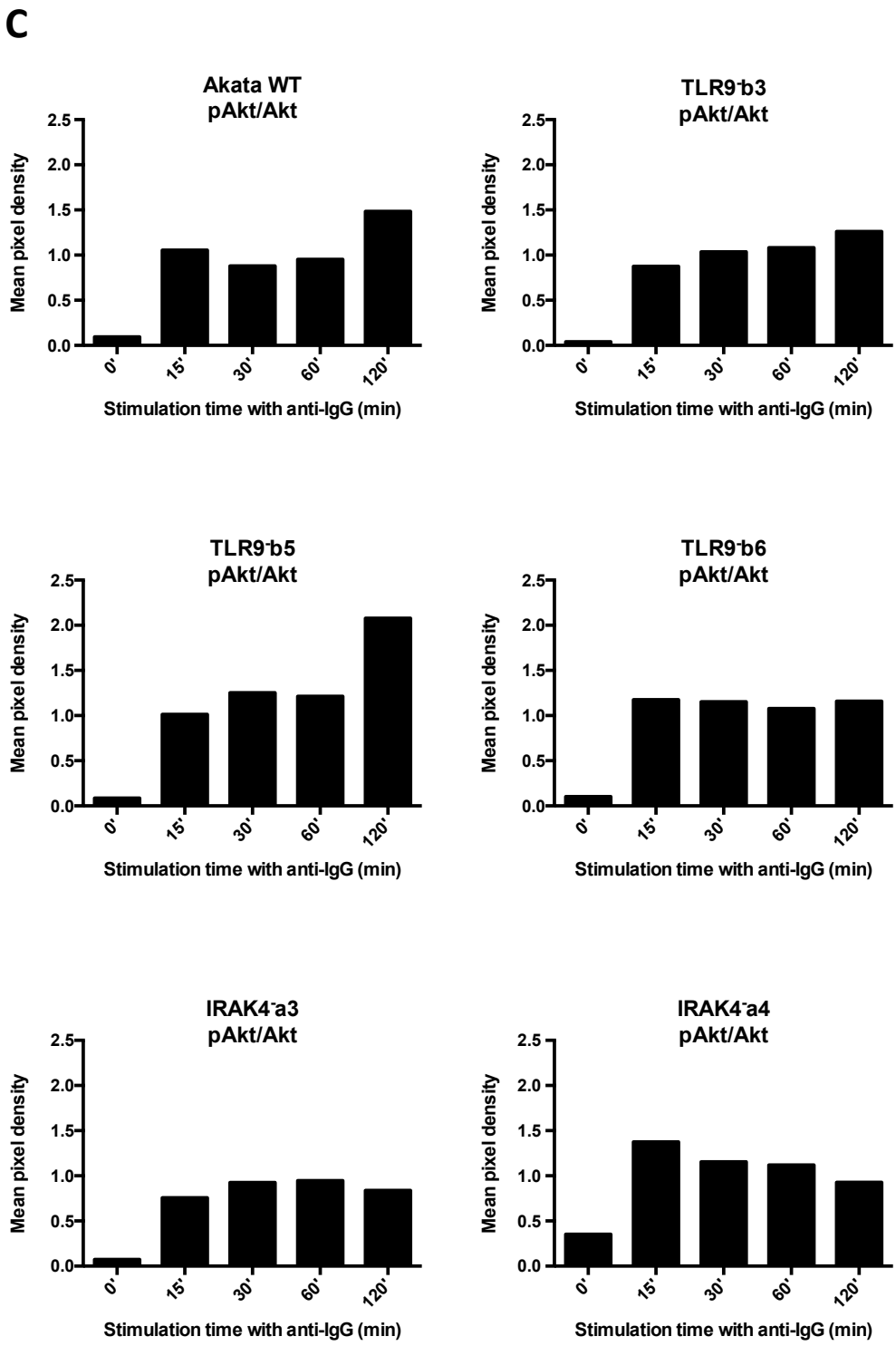


Figure 4: IRAK4 and TLR9 ko do not affect BCR signalling. Akata WT cells and TLR9 (TLR9-b3, TLR9-b5 and TLR9-b6) and IRAK4 (IRAK4-a3 and IRAK4-a4) CRISPR/Cas9 single cell clones were treated with ODN CpG 2006 for 15', 30', 60' and 120'. (A) MEK (B) Syk and (C) Akt phosphorylation was quantified by densitometric analysis. Data are represented as ratio of phosphorylated-to-total. Quantification was performed using the software imageJ 1.49t.

Discussion and outlook

Primary infection of immunocompromised hosts with Epstein-Barr virus (EBV) is associated with an increased risk for EBV-associated tumors. In particular, in the case of immunosuppression related to solid organ and hematopoietic stem cell transplants a clear association has been determined between post-transplant lymphoproliferative disorder (PTLD) development and EBV infection. Despite the crucial role of EBV in the development of PTLD, its etiology still remains obscure. Although risk factors have been identified, it is currently not possible to predict which transplant patients will eventually develop PTLD and why not 100% of the transplant recipients infected with EBV develop EBV-related lymphomas. Thus, we hypothesized that additional mechanisms must be implicated in preventing development of PTLD.

Since species specificity of EBV hampers modeling in experimental animals, we established an *in vitro* model to study EBV B cell infection. Using primary B cells purified from tonsillar mononuclear cells (TMCs) to model primary EBV infection of the EBV naïve host under immunosuppression, we investigated the role of DNA damage response (DDR) pathways in controlling proliferation and transformation of tonsillar B cells (TBCs) during EBV primary infection. In contrast to what has been described so far [64, 144], infection of TBCs with EBV induces expression and activation of the specific proteins ATR and Chk1 in the first 96 hours post inoculation (pi) with increase of pATR over time and a peak of pChk1 activation 72 hours pi. Moreover, and in contrast to what we hypothesized, the specific activation of ATR does not show any antiviral effects but rather seems to increase the ability of EBV to transform B cells *in vitro*. The increased transformation potential of EBV upon activation of the ATR/Chk1 pathway was unexpected. Indeed, transformation of B cells *in vivo*, usually leads to the development of life threatening diseases, such as PTLD, that dramatically decrease the host life span and therefore compromise the persistence of the virus, suggesting that, from a virus point of view, transformation is indeed a mistake.

The discrepancy observed between our work, showing activation of ATR/Chk1, and the work of Nikitin *et al*. [64], showing activation of ATM/Chk2, may have several explanations. Among them, the different origin of B cells, i.e. peripheral

blood B cells (PBMC) vs TBC; the type of EBV strain used for the experiment and the infection protocols, were the most likely to explain the observed differences. Interestingly, in our hands, neither the B cell origin nor the type of EBV strain or the protocol for B cell infection could explain the results at variance that we observed. A deeper analysis of the Luftig's lab protocol suggested that one of the major differences between ours and their model resides in the pool of cells used for the analysis. Indeed, we analyze DDR markers from the total pool of purified infected B cells while they specifically analyze only the proliferating B cells by sorting them by flow cytometry. Given the fact that infected proliferating B cells are only a small percentage of the total purified B cells pool it might be possible that without sorting of the proliferating cells, ATM/Chk2 activation may not be detectable. The higher percentage of dead cells in the total pool of purified cells *versus* the pool of proliferating cells may also be an explanation for the strong ATR/Chk1 activation we observed. Experiments performed with UV inactivated EBV refute this hypothesis and strongly support that activation of ATR/Chk1 is not due to the presence of dying B cells in our analyzed B cell-pool.

EBV (human herpes virus 4) is not the only herpes virus that has been described to induce activation of the ATR/Chk1 pathway. Indeed, human herpes virus 6 (HHV-6), which primarily infects CD4+ T cells, has been shown to induce dramatic levels of cell cycle arrest in G2/M and activation of Chk1 72 hours pi probably in response to increased viral replication stress [100]. Interestingly, infection of CD4+ T cells with HHV-6 has also been shown to induce activation of Chk2. As infection of peripheral B cells (PBC) also induces activation of the ATM/Chk2 pathway and given that ATM/Chk2 and ATR/Chk1 are sequentially triggered when the G2/M checkpoint is activated, we can speculate that in the case of early EBV infection both pathways may be sequentially activated. Thus, depending from the different medical history and genetic background between patient ATM/Chk2 or ATR/Chk1 may prevail and act as an antiviral response against EBV (ATM/Chk2) or as a risk factor increasing EBV dependent B cell transformation (ATR/Chk1), as we observed here.

The robust activation of ATR and Chk1 observed in our model strongly suggests that this pathway plays a crucial role in EBV-driven B-cell transformation in an immunosuppressed environment. Indeed, there is evidence that the ATR/Chk1 pathway could be regulated by c-Myc [145], a transcription factor implicated in a wide range of B-cell malignancies [146]. In a normal context c-Myc is essential for B-cells proliferation and is expressed in subpopulation of germinal center (GC) B-cells

to ensure maintenance of the GC [147, 148]. Given that EBV inoculation of TBCs induces hyperproliferation, a hypothesis could be that c-Myc is overexpressed and responsible for ATR activation increasing the likelihood of inducing EBV-dependent B-cell transformation as well as the probability for the patient to develop an EBV-related B cell lymphoma. Therefore, further experiments could focus on c-Myc expression during the phase of B cell hyperproliferation and on the role that c-Myc might play in inducing activation of the ATR/Chk1 pathway.

To shed light on the mechanism that may lead to the activation of the ATR/Chk1 pathway upon primary EBV infection of TBCs, we used a UV-inactivated form of EBV, able to enter but not to actively infect B cells, as well as a combination of CD40L/IL-4 known to induce B cell hyperproliferation [149]. Our results illustrate how a phase of active hyperproliferation is sufficient and necessary to induce activation of ATR/Chk1 in B cells. On the other hand, induction of hyperproliferation *via* CD40L/IL-4 stimulation is not sufficient to induce B cell transformation. Therefore, we can conclude that enhanced risk of EBV dependent B cell transformation in immunosuppressed patients needs both EBV and additional stimuli that will trigger hyperproliferation of the infected B cells.

Albeit our results suggest an important role played by ATR but not by Chk1 in the development of EBV related PTLD, we still cannot explain why not 100% of the transplanted patients will develop EBV related lymphomas following EBV primary infection. We hypothesize that previous bacterial or viral infections might stimulate TBCs proliferation leading to replication stress and consequently activation of the ATR/Chk1-mediated DDR, which will increase the probability of EBV transformation. Indeed, it is known that TLRs signaling, including TLR9 that bind specific common patterns on bacterial DNA (CpG motifs), can directly induce B-cell proliferation [149, 150]. Furthermore, it has been shown in our lab that triggering of TLR9 signaling in a Burkitt's lymphoma derived cell line blocks reactivation of EBV lytic cycle, reinforcing the risk of cell transformation [39]. Further factors may also be required *in vivo* in addition to ATR to significantly increase the risk of EBV B cell transformation. These factors might be expressed only by certain individuals and therefore explain why PTLD is not more prevalent among immunosuppressed patients (**Figure 5**).

Taken together, our results emphasize the importance of DDR during EBV primary infection and show, for the first time, how EBV might hijack a cellular mechanism that facilitates EBV-dependent B cell transformation. The identification of

ATR as the responsible key factor for this erroneous transformation of B cells, suggests that targeting of the interaction between ATR and EBV could offer new therapeutic options for the treatment of EBV-associated malignancies.

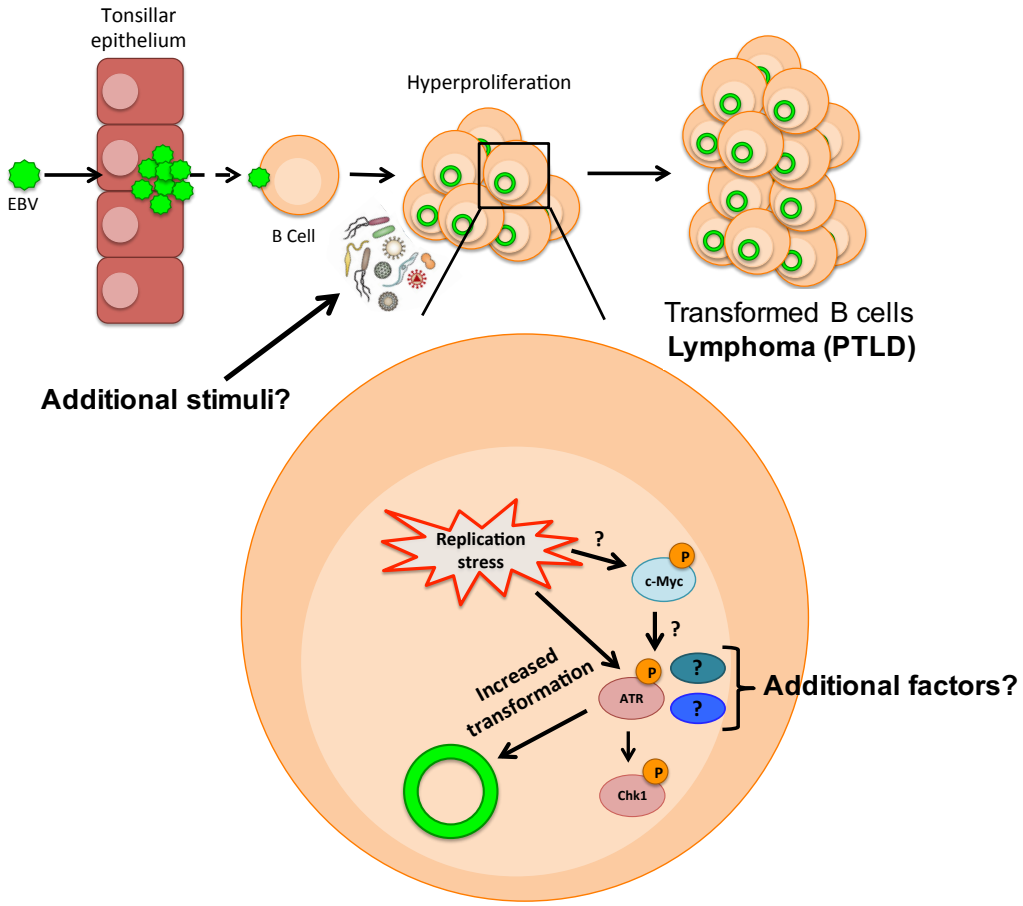


Figure 5: Model for the role of DDR in enhancing B cell transformation in immune-compromised patients. EBV enters its host by infecting tonsillar epithelial cells in the oropharynx where it establishes a primary focus of lytic replication. From the tonsillar epithelium, the newly synthesized virions will spread through the lymphoid tissues as a latent growth-transforming infection (latency III) of B cells. Following this phase of hyperproliferation EBV infection can lead to the development of PTLD. We hypothesized that additional stimuli, such as previous bacterial or viral infections, might stimulate TBCs proliferation. This would lead to replication stress and consequently to activation of the ATR/Chk1-mediated DDR, either directly or through c-Myc signaling. This would result in an increase of the transformation potential of the virus and of the risk of developing lymphomas. Additional factors might also interact directly with ATR increasing the chances for EBV to transform B cells.

Acknowledgment

My PhD thesis would not have been possible without the help and support of many people. I would like to express my great gratitude to everyone who supported and helped me during this four years as a PhD student, in particular:

Prof. Dr. David Nadal

Who gave me the opportunity to work on this challenging project in his Lab. Thank you for your motivation, enthusiasm, and immense knowledge. For always being supportive and open to discussion and for guiding me through my PhD always being able to bring out the best out of me.

PD Dr. Michele Bernasconi

Thank you so much for all your support and patient that helped me to successfully work through my PhD. Your door was always open to discuss scientific and non-scientific matter and for a coffee. Last but not least, you were always present to motivate me when I needed some encouragement.

Prof. Dr. Christian Münz; Prof. Dr. Anne Müller; Prof. Dr. Alessandro Sartori; Prof. Dr. Cornel Fraefel

Thank you for being members of my thesis committee, for taking your time to judge my yearly progress and for the stimulating suggestions on my project.

Former and present members of the infectiology group: Florian Haas; Jürgen Rac; Patricia Krukoswki; Tarik Azzi; Jeannine Marty; Anna Lünneman; Simone Bürgler; Marc Jordi; Sabrina Traxel; Semjon Siderov; Patrick Meyer; Monika Kotur; Isabelle Treichler; Aurelia Jud

It was a pleasure to be part of such a fantastic group and to share all these happy and sometimes frustrating moments I experienced during my four year of PhD.

All the present and former members I got to know of the oncology and leukemia group

Thank you for the time we spent together discussing, celebrating, chatting and laughing. You made these four years I spent in AFS unforgettable.

A special thanks goes to my friend and colleague Chiara Giorgi

Thank you for you support and friendship. This for years would not have been the same without the best office-mate I could wish for.

My parents

Christine and Marco, for always being supportive whatever I decided to do. I would not be the person I am today if it were not for you and for this I will always be grateful.

Patrizio

Thank you for always being present when I need support, for always listening when I need a confident, for always believing in me and for always being able to make me laughs when I am down.

Literature

1. Beishline K and Azizkhan-Clifford J. Interplay between the cell cycle and double-strand break response in mammalian cells. Methods Mol Biol. 2014; 1170:41-59.
2. Epstein MA, Achong BG and Barr YM. Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma. Lancet. 1964; 1(7335):702-703.
3. Fields B, Knipe D and Howley P. (2007). Epstein Barr virus. Fields Virology. (Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins).
4. Thorley-Lawson DA. Epstein-Barr virus: exploiting the immune system. Nat Rev Immunol. 2001; 1(1):75-82.
5. Krabbe S, Hesse J and Uldall P. Primary Epstein-Barr virus infection in early childhood. Arch Dis Child. 1981; 56(1):49-52.
6. Dunmire S, Hogquist K and Balfour H. Infectious Mononucleosis. Current topics in microbiology and immunology 2015; 390:211-240.
7. Sample J and Sample C. (2008). Epstein-Barr Virus: Molecular Biology. In: Mahy BWJ and Van Regenmorte MHV, eds. Encyclopedia of virologi: Academic Press).
8. Skalsky RL and Cullen BR. (2015). EBV Noncodinf RNAs. In: C.Münz, ed. Epstein Barr Virus Volume 2. (Switzerland: Springer International Publishing Switzerland).
9. Countryman J, Jenson H, Seibl R, Wolf H and Miller G. Polymorphic proteins encoded within BZLF1 of defective and standard Epstein-Barr viruses disrupt latency. J Virol. 1987; 61(12):3672-3679.
10. Tovey MG, Lenoir G and Begon-Lours J. Activation of latent Epstein-Barr virus by antibody to human IgM. Nature. 1978; 276(5685):270-272.
11. zur Hausen H, O'Neill FJ, Freese UK and Hecker E. Persisting oncogenic herpesvirus induced by the tumour promotor TPA. Nature. 1978; 272(5651):373-375.
12. Luka J, Kallin B and Klein G. Induction of the Epstein-Barr virus (EBV) cycle in latently infected cells by n-butyrate. Virology. 1979; 94(1):228-231.
13. Takada K. Cross-linking of cell surface immunoglobulins induces Epstein-Barr virus in Burkitt lymphoma lines. Int J Cancer. 1984; 33(1):27-32.
14. di Renzo L, Altiok A, Klein G and Klein E. Endogenous TGF-beta contributes to the induction of the EBV lytic cycle in two Burkitt lymphoma cell lines. Int J Cancer. 1994; 57(6):914-919.
15. Aitken C, Sengupta SK, Aedes C, Moss DJ and Sculley TB. Heterogeneity within the Epstein-Barr virus nuclear antigen 2 gene in different strains of Epstein-Barr virus. J Gen Virol. 1994; 75 (Pt 1):95-100.
16. Sample J, Young L, Martin B, Chatman T, Kieff E, Rickinson A and Kieff E. Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes. J Virol. 1990; 64(9):4084-4092.
17. Tzellos S and Farrell PJ. Epstein-barr virus sequence variation-biology and disease. Pathogens. 2012; 1(2):156-174.
18. Thorley-Lawson DA and Gross A. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. N Engl J Med. 2004; 350(13):1328-1337.
19. Yajima M, Kanda T and Takada K. Critical role of Epstein-Barr Virus (EBV)-encoded RNA in efficient EBV-induced B-lymphocyte growth transformation. J Virol. 2005; 79(7):4298-4307.
20. Wu Y, Maruo S, Yajima M, Kanda T and Takada K. Epstein-Barr virus (EBV)-encoded RNA 2 (EBER2) but not EBER1 plays a critical role in EBV-induced B-cell growth transformation. J Virol. 2007; 81(20):11236-11245.

21. Gregorovic G, Bosshard R, Karstegl CE, White RE, Pattle S, Chiang AK, Dittrich-Breiholz O, Kracht M, Russ R and Farrell PJ. Cellular gene expression that correlates with EBER expression in Epstein-Barr Virus-infected lymphoblastoid cell lines. J Virol. 2011; 85(7):3535-3545.
22. Repellin CE, Tsimbouri PM, Philbey AW and Wilson JB. Lymphoid hyperplasia and lymphoma in transgenic mice expressing the small non-coding RNA, EBER1 of Epstein-Barr virus. PLoS One. 2010; 5(2):e9092.
23. Swaminathan S, Tomkinson B and Kieff E. Recombinant Epstein-Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates in vitro. Proc Natl Acad Sci U S A. 1991; 88(4):1546-1550.
24. Babcock GJ, Decker LL, Volk M and Thorley-Lawson DA. EBV persistence in memory B cells in vivo. Immunity. 1998; 9(3):395-404.
25. Kaiser C, Laux G, Eick D, Jochner N, Bornkamm GW and Kempkes B. The proto-oncogene c-myc is a direct target gene of Epstein-Barr virus nuclear antigen 2. J Virol. 1999; 73(5):4481-4484.
26. Sinclair AJ, Palmero I, Peters G and Farrell PJ. EBNA-2 and EBNA-LP cooperate to cause G0 to G1 transition during immortalization of resting human B lymphocytes by Epstein-Barr virus. EMBO J. 1994; 13(14):3321-3328.
27. Thorley-Lawson DA and Babcock GJ. A model for persistent infection with Epstein-Barr virus: the stealth virus of human B cells. Life Sci. 1999; 65(14):1433-1453.
28. Kuppers R. B cells under influence: transformation of B cells by Epstein-Barr virus. Nat Rev Immunol. 2003; 3(10):801-812.
29. Hochberg D, Middeldorp JM, Catalina M, Sullivan JL, Luzuriaga K and Thorley-Lawson DA. Demonstration of the Burkitt's lymphoma Epstein-Barr virus phenotype in dividing latently infected memory cells in vivo. Proc Natl Acad Sci U S A. 2004; 101(1):239-244.
30. Young LS and Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer. 2004; 4(10):757-768.
31. Dorner M, Zucol F, Berger C, Byland R, Melroe GT, Bernasconi M, Speck RF and Nadal D. Distinct ex vivo susceptibility of B-cell subsets to epstein-barr virus infection according to differentiation status and tissue origin. J Virol. 2008; 82(9):4400-4412.
32. Babcock GJ, Hochberg D and Thorley-Lawson AD. The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell. Immunity. 2000; 13(4):497-506.
33. Murphy K. (2011). Janeway's Immunobiology. (New York: Garland science).
34. Ishii KJ, Koyama S, Nakagawa A, Coban C and Akira S. Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe. 2008; 3(6):352-363.
35. Dorner M, Brandt S, Tinguely M, Zucol F, Bourquin JP, Zauner L, Berger C, Bernasconi M, Speck RF and Nadal D. Plasma cell toll-like receptor (TLR) expression differs from that of B cells, and plasma cell TLR triggering enhances immunoglobulin production. Immunology. 2009; 128(4):573-579.
36. Heeg K, Dalpke A, Peter M and Zimmermann S. Structural requirements for uptake and recognition of CpG oligonucleotides. Int J Med Microbiol. 2008; 298(1-2):33-38.
37. van Gent M, Griffin BD, Berkhoff EG, van Leeuwen D, Boer IG, Buisson M, Hartgers FC, Burmeister WP, Wiertz EJ and Ressing ME. EBV lytic-phase protein BGLF5 contributes to TLR9 downregulation during productive infection. J Immunol. 2011; 186(3):1694-1702.
38. Takeshita F, Gursel I, Ishii KJ, Suzuki K, Gursel M and Klinman DM. Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9. Semin Immunol. 2004; 16(1):17-22.
39. Zauner L, Melroe GT, Sigrist JA, Rechsteiner MP, Dorner M, Arnold M, Berger C, Bernasconi M, Schaefer BW, Speck RF and Nadal D. TLR9 triggering in Burkitt's lymphoma cell lines suppresses the EBV BZLF1 transcription via histone modification. Oncogene. 2010; 29(32):4588-4598.
40. Weiss LM, Strickler JG, Warnke RA, Purtilo DT and Sklar J. Epstein-Barr viral DNA in tissues of Hodgkin's disease. Am J Pathol. 1987; 129(1):86-91.
41. Hanto DW, Gajl-Peczalska KJ, Frizzera G, Arthur DC, Balfour HH, Jr., McClain K, Simmons RL and Najarian JS. Epstein-Barr virus (EBV) induced polyclonal and monoclonal B-cell

lymphoproliferative diseases occurring after renal transplantation. Clinical, pathologic, and virologic findings and implications for therapy. Ann Surg. 1983; 198(3):356-369.
42. Ho M, Miller G, Atchison RW, Breinig MK, Dummer JS, Andiman W, Starzl TE, Eastman R, Griffith BP, Hardesty RL and et al. Epstein-Barr virus infections and DNA hybridization studies in posttransplantation lymphoma and lymphoproliferative lesions: the role of primary infection. J Infect Dis. 1985; 152(5):876-886.
43. Paya CV, Fung JJ, Nalesnik MA, Kieff E, Green M, Gores G, Habermann TM, Wiesner PH, Swinnen JL, Woodle ES and Bromberg JS. Epstein-Barr virus-induced posttransplant lymphoproliferative disorders. ASTS/ASTP EBV-PTLD Task Force and The Mayo Clinic Organized International Consensus Development Meeting. Transplantation. 1999; 68(10):1517-1525.
44. Taylor GS, Long HM, Brooks JM, Rickinson AB and Hislop AD. The immunology of Epstein-Barr virus-induced disease. Annu Rev Immunol. 2015; 33:787-821.
45. Sugden B and Mark W. Clonal transformation of adult human leukocytes by Epstein-Barr virus. J Virol. 1977; 23(3):503-508.
46. Aman P, Ehlin-Henriksson B and Klein G. Epstein-Barr virus susceptibility of normal human B lymphocyte populations. J Exp Med. 1984; 159(1):208-220.
47. Rowe M, Lear AL, Croom-Carter D, Davies AH and Rickinson AB. Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. J Virol. 1992; 66(1):122-131.
48. Young LS, Sung NS and Pagano JS. (2009). Epstein-Barr Virus. eLS.
49. Jones JF, Shurin S, Abramowsky C, Tubbs RR, Sciotto CG, Wahl R, Sands J, Gottman D, Katz BZ and Sklar J. T-cell lymphomas containing Epstein-Barr viral DNA in patients with chronic Epstein-Barr virus infections. N Engl J Med. 1988; 318(12):733-741.
50. Harabuchi Y, Yamanaka N, Kataura A, Imai S, Kinoshita T, Mizuno F and Osato T. Epstein-Barr virus in nasal T-cell lymphomas in patients with lethal midline granuloma. Lancet. 1990; 335(8682):128-130.
51. Abdel-Hamid M, Chen JJ, Constantine N, Massoud M and Raab-Traub N. EBV strain variation: geographical distribution and relation to disease state. Virology. 1992; 190(1):168-175.
52. Penn I, Hammond W, Brettschneider L and Starzl TE. Malignant lymphomas in transplantation patients. Transplant Proc. 1969; 1(1):106-112.
53. Taylor AL, Marcus R and Bradley JA. Post-transplant lymphoproliferative disorders (PTLD) after solid organ transplantation. Crit Rev Oncol Hematol. 2005; 56(1):155-167.
54. Leblond V, Sutton L, Dorent R, Davi F, Bitker MO, Gabarre J, Charlotte F, Ghoussoub JJ, Fourcade C, Fischer A and et al. Lymphoproliferative disorders after organ transplantation: a report of 24 cases observed in a single center. J Clin Oncol. 1995; 13(4):961-968.
55. Morrison VA, Dunn DL, Manivel JC, Gajl-Peczalska KJ and Peterson BA. Clinical characteristics of post-transplant lymphoproliferative disorders. Am J Med. 1994; 97(1):14-24.
56. Kwong YL, Lam CC and Chan TM. Post-transplantation lymphoproliferative disease of natural killer cell lineage: a clinicopathological and molecular analysis. Br J Haematol. 2000; 110(1):197-202.
57. Lau LG, Tan LK, Salto-Tellez M, Koay ES and Liu TC. T-cell post-transplant lymphoproliferative disorder after hematopoietic stem cell transplantation: another case and a review of the literature. Bone Marrow Transplant. 2004; 34(9):821-822.
58. Purtilo DT. Epstein-Barr-virus-induced oncogenesis in immune-deficient individuals. Lancet. 1980; 1(8163):300-303.
59. Mynarek M, Schober T, Behrends U and Maecker-Kolhoff B. Posttransplant lymphoproliferative disease after pediatric solid organ transplantation. Clin Dev Immunol. 2013; 2013:814973.
60. Feng S, Buell JF, Chari RS, DiMaio JM and Hanto DW. Tumors and transplantation: The 2003 Third Annual ASTS State-of-the-Art Winter Symposium. Am J Transplant. 2003; 3(12):1481-1487.
61. Boubenider S, Hiesse C, Goupy C, Kriaa F, Marchand S and Charpentier B. Incidence and consequences of post-transplantation lymphoproliferative disorders. J Nephrol. 1997; 10(3):136-145.

62. Dolcetti R. B lymphocytes and Epstein-Barr virus: the lesson of post-transplant lymphoproliferative disorders. Autoimmun Rev. 2007; 7(2):96-101.
63. Opelz G and Dohler B. Lymphomas after solid organ transplantation: a collaborative transplant study report. Am J Transplant. 2004; 4(2):222-230.
64. Nikitin PA, Yan CM, Forte E, Bocedi A, Tourigny JP, White RE, Allday MJ, Patel A, Dave SS, Kim W, Hu K, Guo J, Tainter D, Rusyn E and Luftig MA. An ATM/Chk2-mediated DNA damage-responsive signaling pathway suppresses Epstein-Barr virus transformation of primary human B cells. Cell Host Microbe. 2010; 8(6):510-522.
65. Ciccia A and Elledge SJ. The DNA damage response: making it safe to play with knives. Mol Cell. 2010; 40(2):179-204.
66. Hoeijmakers JH. DNA damage, aging, and cancer. N Engl J Med. 2009; 361(15):1475-1485.
67. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev. 2001; 15(17):2177-2196.
68. Wright JA, Keegan KS, Herendeen DR, Bentley NJ, Carr AM, Hoekstra MF and Concannon P. Protein kinase mutants of human ATR increase sensitivity to UV and ionizing radiation and abrogate cell cycle checkpoint control. Proc Natl Acad Sci U S A. 1998; 95(13):7445-7450.
69. Guo Z, Kumagai A, Wang SX and Dunphy WG. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in Xenopus egg extracts. Genes Dev. 2000; 14(21):2745-2756.
70. Zou L and Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science. 2003; 300(5625):1542-1548.
71. Bartek J and Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell. 2003; 3(5):421-429.
72. Stracker TH, Usui T and Petrini JH. Taking the time to make important decisions: the checkpoint effector kinases Chk1 and Chk2 and the DNA damage response. DNA Repair (Amst). 2009; 8(9):1047-1054.
73. Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA and Elledge SJ. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev. 2000; 14(12):1448-1459.
74. Zhao H and Piwnica-Worms H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol. 2001; 21(13):4129-4139.
75. Delacroix S, Wagner JM, Kobayashi M, Yamamoto K and Karnitz LM. The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1. Genes Dev. 2007; 21(12):1472-1477.
76. Kumagai A, Lee J, Yoo HY and Dunphy WG. TopBP1 activates the ATR-ATRIP complex. Cell. 2006; 124(5):943-955.
77. Jeong SY, Kumagai A, Lee J and Dunphy WG. Phosphorylated claspin interacts with a phosphate-binding site in the kinase domain of Chk1 during ATR-mediated activation. J Biol Chem. 2003; 278(47):46782-46788.
78. Kumagai A and Dunphy WG. Repeated phosphopeptide motifs in Claspin mediate the regulated binding of Chk1. Nat Cell Biol. 2003; 5(2):161-165.
79. Bakkenist CJ and Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature. 2003; 421(6922):499-506.
80. Uziel T, Lerenthal Y, Moyal L, Andegeko Y, Mittelman L and Shiloh Y. Requirement of the MRN complex for ATM activation by DNA damage. EMBO J. 2003; 22(20):5612-5621.
81. Lee JH and Paull TT. Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. Science. 2004; 304(5667):93-96.
82. Rogakou EP, Boon C, Redon C and Bonner WM. Megabase chromatin domains involved in DNA double-strand breaks in vivo. J Cell Biol. 1999; 146(5):905-916.
83. Polo SE and Jackson SP. Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications. Genes Dev. 2011; 25(5):409-433.

84. Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K and Elledge SJ. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc Natl Acad Sci U S A. 2000; 97(19):10389-10394.
85. Lukas C, Falck J, Bartkova J, Bartek J and Lukas J. Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage. Nat Cell Biol. 2003; 5(3):255-260.
86. Ziv Y, Bielopolski D, Galanty Y, Lukas C, Taya Y, Schultz DC, Lukas J, Bekker-Jensen S, Bartek J and Shiloh Y. Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway. Nat Cell Biol. 2006; 8(8):870-876.
87. Iyengar S and Farnham PJ. KAP1 protein: an enigmatic master regulator of the genome. J Biol Chem. 2011; 286(30):26267-26276.
88. Noon AT, Shibata A, Rief N, Lobrich M, Stewart GS, Jeggo PA and Goodarzi AA. 53BP1-dependent robust localized KAP-1 phosphorylation is essential for heterochromatic DNA double-strand break repair. Nat Cell Biol. 2010; 12(2):177-184.
89. White D, Rafalska-Metcalf IU, Ivanov AV, Corsinotti A, Peng H, Lee SC, Trono D, Janicki SM and Rauscher FJ, 3rd. The ATM substrate KAP1 controls DNA repair in heterochromatin: regulation by HP1 proteins and serine 473/824 phosphorylation. Mol Cancer Res. 2012; 10(3):401-414.
90. Smith J, Tho LM, Xu N and Gillespie DA. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. Adv Cancer Res. 2010; 108:73-112.
91. Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H and Elledge SJ. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science. 1997; 277(5331):1497-1501.
92. Hirao A, Kong YY, Matsuoka S, Wakeham A, Ruland J, Yoshida H, Liu D, Elledge SJ and Mak TW. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science. 2000; 287(5459):1824-1827.
93. Mazouzi A, Velimezi G and Loizou JI. DNA replication stress: causes, resolution and disease. Exp Cell Res. 2014; 329(1):85-93.
94. Sorensen CS, Syljuasen RG, Falck J, Schroeder T, Ronnstrand L, Khanna KK, Zhou BB, Bartek J and Lukas J. Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A. Cancer Cell. 2003; 3(3):247-258.
95. Lobrich M and Jeggo PA. The impact of a negligent G2/M checkpoint on genomic instability and cancer induction. Nat Rev Cancer. 2007; 7(11):861-869.
96. Lilley CE, Chaurushiya MS, Boutell C, Everett RD and Weitzman MD. The intrinsic antiviral defense to incoming HSV-1 genomes includes specific DNA repair proteins and is counteracted by the viral protein ICP0. PLoS Pathog. 2011; 7(6):e1002084.
97. Lilley CE, Carson CT, Muotri AR, Gage FH and Weitzman MD. DNA repair proteins affect the lifecycle of herpes simplex virus 1. Proc Natl Acad Sci U S A. 2005; 102(16):5844-5849.
98. Wilkinson DE and Weller SK. Recruitment of cellular recombination and repair proteins to sites of herpes simplex virus type 1 DNA replication is dependent on the composition of viral proteins within prereplicative sites and correlates with the induction of the DNA damage response. J Virol. 2004; 78(9):4783-4796.
99. Shirata N, Kudoh A, Daikoku T, Tatsumi Y, Fujita M, Kiyono T, Sugaya Y, Isomura H, Ishizaki K and Tsurumi T. Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. J Biol Chem. 2005; 280(34):30336-30341.
100. Li L, Gu B, Zhou F, Chi J, Wang F, Peng G, Xie F, Qing J, Feng D, Lu S and Yao K. Human herpesvirus 6 suppresses T cell proliferation through induction of cell cycle arrest in infected cells in the G2/M phase. J Virol. 2011; 85(13):6774-6783.
101. Singh VV, Dutta D, Ansari MA, Dutta S and Chandran B. Kaposi's sarcoma-associated herpesvirus induces the ATM and H2AX DNA damage response early during de novo infection of primary endothelial cells, which play roles in latency establishment. J Virol. 2014; 88(5):2821-2834.
102. Wang'ondu R, Teal S, Park R, Heston L, Delecluse H and Miller G. DNA Damage Signaling Is Induced in the Absence of Epstein-Barr Virus (EBV) Lytic DNA Replication and in Response to Expression of ZEBRA. Plos One. 2015; 10(5).

103. Li R, Liao G, Nirujogi RS, Pinto SM, Shaw PG, Huang TC, Wan J, Qian J, Gowda H, Wu X, Lv DW, Zhang K, Manda SS, Pandey A and Hayward SD. Phosphoproteomic Profiling Reveals Epstein-Barr Virus Protein Kinase Integration of DNA Damage Response and Mitotic Signaling. PLoS Pathog. 2015; 11(12):e1005346.
104. Jha HC, Prasad AJM, Saha A, Banerjee S, Lu J and Robertson ES. Epstein-Barr Virus Essential Antigen EBNA3C Attenuates H2AX Expression. Journal of Virology. 2014; 88(7):3776-3788.
105. Boichuk S, Hu L, Hein J and Gjoerup OV. Multiple DNA damage signaling and repair pathways deregulated by simian virus 40 large T antigen. J Virol. 2010; 84(16):8007-8020.
106. Shi Y, Dodson GE, Shaikh S, Rundell K and Tibbetts RS. Ataxia-telangiectasia-mutated (ATM) is a T-antigen kinase that controls SV40 viral replication in vivo. J Biol Chem. 2005; 280(48):40195-40200.
107. Moody CA and Laimins LA. Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation. PLoS Pathog. 2009; 5(10):e1000605.
108. E X, Pickering MT, Debatis M, Castillo J, Lagadinos A, Wang S, Lu S and Kowalik TF. An E2F1-mediated DNA damage response contributes to the replication of human cytomegalovirus. PLoS Pathog. 2011; 7(5):e1001342.
109. Shah GA and O'Shea CC. Viral and Cellular Genomes Activate Distinct DNA Damage Responses. Cell. 2015; 162(5):987-1002.
110. Skalka AM and Katz RA. Retroviral DNA integration and the DNA damage response. Cell Death Differ. 2005; 12 Suppl 1:971-978.
111. Stracker TH, Carson CT and Weitzman MD. Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. Nature. 2002; 418(6895):348-352.
112. Baker A, Rohleder KJ, Hanakahi LA and Ketner G. Adenovirus E4 34k and E1b 55k oncoproteins target host DNA ligase IV for proteasomal degradation. J Virol. 2007; 81(13):7034-7040.
113. Blackford AN, Patel RN, Forrester NA, Theil K, Groitl P, Stewart GS, Taylor AM, Morgan IM, Dobner T, Grand RJ and Turnell AS. Adenovirus 12 E4orf6 inhibits ATR activation by promoting TOPBP1 degradation. Proc Natl Acad Sci U S A. 2010; 107(27):12251-12256.
114. Parkinson J, Lees-Miller SP and Everett RD. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. J Virol. 1999; 73(1):650-657.
115. Chaurushiya MS, Lilley CE, Aslanian A, Meisenhelder J, Scott DC, Landry S, Ticau S, Boutell C, Yates JR, 3rd, Schulman BA, Hunter T and Weitzman MD. Viral E3 ubiquitin ligase-mediated degradation of a cellular E3: viral mimicry of a cellular phosphorylation mark targets the RNF8 FHA domain. Mol Cell. 2012; 46(1):79-90.
116. Lilley CE, Chaurushiya MS, Boutell C, Landry S, Suh J, Panier S, Everett RD, Stewart GS, Durocher D and Weitzman MD. A viral E3 ligase targets RNF8 and RNF168 to control histone ubiquitination and DNA damage responses. EMBO J. 2010; 29(5):943-955.
117. Mohni KN, Smith S, Dee AR, Schumacher AJ and Weller SK. Herpes simplex virus type 1 single strand DNA binding protein and helicase/primase complex disable cellular ATR signaling. PLoS Pathog. 2013; 9(10):e1003652.
118. Ou HD, Kwiatkowski W, Deerinck TJ, Noske A, Blain KY, Land HS, Soria C, Powers CJ, May AP, Shu X, Tsien RY, Fitzpatrick JA, Long JA, Ellisman MH, Choe S and O'Shea CC. A structural basis for the assembly and functions of a viral polymer that inactivates multiple tumor suppressors. Cell. 2012; 151(2):304-319.
119. Carson CT, Orazio NI, Lee DV, Suh J, Bekker-Jensen S, Araujo FD, Lakdawala SS, Lilley CE, Bartek J, Lukas J and Weitzman MD. Mislocalization of the MRN complex prevents ATR signaling during adenovirus infection. EMBO J. 2009; 28(6):652-662.
120. Liang X, Pickering MT, Cho NH, Chang H, Volkert MR, Kowalik TF and Jung JU. Deregulation of DNA damage signal transduction by herpesvirus latency-associated M2. J Virol. 2006; 80(12):5862-5874.

121. Shin YC, Nakamura H, Liang X, Feng P, Chang H, Kowalik TF and Jung JU. Inhibition of the ATM/p53 signal transduction pathway by Kaposi's sarcoma-associated herpesvirus interferon regulatory factor 1. J Virol. 2006; 80(5):2257-2266.
122. Lai CK, Jeng KS, Machida K, Cheng YS and Lai MM. Hepatitis C virus NS3/4A protein interacts with ATM, impairs DNA repair and enhances sensitivity to ionizing radiation. Virology. 2008; 370(2):295-309.
123. Schmid M, Speiseder T, Dobner T and Gonzalez RA. DNA virus replication compartments. J Virol. 2014; 88(3):1404-1420.
124. Luftig MA. Viruses and the DNA Damage Response: Activation and Antagonism. Annu Rev Virol. 2014; 1(1):605-625.
125. Taylor TJ and Knipe DM. Proteomics of herpes simplex virus replication compartments: association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. J Virol. 2004; 78(11):5856-5866.
126. Hagemeier SR, Barlow EA, Meng Q and Kenney SC. The cellular ataxia telangiectasia-mutated kinase promotes epstein-barr virus lytic reactivation in response to multiple different types of lytic reactivation-inducing stimuli. J Virol. 2012; 86(24):13360-13370.
127. Daikoku T, Kudoh A, Sugaya Y, Iwahori S, Shirata N, Isomura H and Tsurumi T. Postreplicative mismatch repair factors are recruited to Epstein-Barr virus replication compartments. J Biol Chem. 2006; 281(16):11422-11430.
128. Casavant NC, Luo MH, Rosenke K, Winegardner T, Zurawska A and Fortunato EA. Potential role for p53 in the permissive life cycle of human cytomegalovirus. J Virol. 2006; 80(17):8390-8401.
129. Bagga S and Bouchard MJ. Cell cycle regulation during viral infection. Methods Mol Biol. 2014; 1170:165-227.
130. Henderson E, Miller G, Robinson J and Heston L. Efficiency of transformation of lymphocytes by Epstein-Barr virus. Virology. 1977; 76(1):152-163.
131. Price AM, Tourigny JP, Forte E, Salinas RE, Dave SS and Luftig MA. Analysis of Epstein-Barr virus-regulated host gene expression changes through primary B-cell outgrowth reveals delayed kinetics of latent membrane protein 1-mediated NF-kappaB activation. J Virol. 2012; 86(20):11096-11106.
132. Forte E, Salinas RE, Chang C, Zhou T, Linnstaedt SD, Gottwein E, Jacobs C, Jima D, Li QJ, Dave SS and Luftig MA. The Epstein-Barr virus (EBV)-induced tumor suppressor microRNA MiR-34a is growth promoting in EBV-infected B cells. J Virol. 2012; 86(12):6889-6898.
133. Choudhuri T, Verma SC, Lan K, Murakami M and Robertson ES. The ATM/ATR signaling effector Chk2 is targeted by Epstein-Barr virus nuclear antigen 3C to release the G2/M cell cycle block. J Virol. 2007; 81(12):6718-6730.
134. Skalska L, White RE, Franz M, Ruhmann M and Allday MJ. Epigenetic repression of p16(INK4A) by latent Epstein-Barr virus requires the interaction of EBNA3A and EBNA3C with CtBP. PLoS Pathog. 2010; 6(6):e1000951.
135. Rodier F, Munoz DP, Teachenor R, Chu V, Le O, Bhaumik D, Coppe JP, Campeau E, Beausejour CM, Kim SH, Davalos AR and Campisi J. DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. J Cell Sci. 2011; 124(Pt 1):68-81.
136. Gruhne B, Sompallae R and Masucci MG. Three Epstein-Barr virus latency proteins independently promote genomic instability by inducing DNA damage, inhibiting DNA repair and inactivating cell cycle checkpoints. Oncogene. 2009; 28(45):3997-4008.
137. Zhou J, Deng Z, Norseen J and Lieberman PM. Regulation of Epstein-Barr virus origin of plasmid replication (OriP) by the S-phase checkpoint kinase Chk2. J Virol. 2010; 84(10):4979-4987.
138. Dheekollu J, Deng Z, Wiedmer A, Weitzman MD and Lieberman PM. A role for MRE11, NBS1, and recombination junctions in replication and stable maintenance of EBV episomes. PLoS One. 2007; 2(12):e1257.

139. Sun Y, Jiang X, Chen S, Fernandes N and Price BD. A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. Proc Natl Acad Sci U S A. 2005; 102(37):13182-13187.
140. Kudoh A, Fujita M, Zhang L, Shirata N, Daikoku T, Sugaya Y, Isomura H, Nishiyama Y and Tsurumi T. Epstein-Barr virus lytic replication elicits ATM checkpoint signal transduction while providing an S-phase-like cellular environment. J Biol Chem. 2005; 280(9):8156-8163.
141. Li R, Zhu J, Xie Z, Liao G, Liu J, Chen MR, Hu S, Woodard C, Lin J, Taverna SD, Desai P, Ambinder RF, Hayward GS, Qian J, Zhu H and Hayward SD. Conserved herpesvirus kinases target the DNA damage response pathway and TIP60 histone acetyltransferase to promote virus replication. Cell Host Microbe. 2011; 10(4):390-400.
142. Kudoh A, Fujita M, Kiyono T, Kuzushima K, Sugaya Y, Izuta S, Nishiyama Y and Tsurumi T. Reactivation of lytic replication from B cells latently infected with Epstein-Barr virus occurs with high S-phase cyclin-dependent kinase activity while inhibiting cellular DNA replication. J Virol. 2003; 77(2):851-861.
143. Flemington EK. Herpesvirus lytic replication and the cell cycle: arresting new developments. J Virol. 2001; 75(10):4475-4481.
144. Koganti S, Hui-Yuen J, McAllister S, Gardner B, Grasser F, Palendira U, Tangye SG, Freeman AF and Bhaduri-McIntosh S. STAT3 interrupts ATR-Chk1 signaling to allow oncovirus-mediated cell proliferation. Proc Natl Acad Sci U S A. 2014; 111(13):4946-4951.
145. Campaner S and Amati B. Two sides of the Myc-induced DNA damage response: from tumor suppression to tumor maintenance. Cell Div. 2012; 7(1):6.
146. Klein U and Dalla-Favera R. Germinal centres: role in B-cell physiology and malignancy. Nat Rev Immunol. 2008; 8(1):22-33.
147. de Alboran IM, O'Hagan RC, Gartner F, Malynn B, Davidson L, Rickert R, Rajewsky K, DePinho RA and Alt FW. Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. Immunity. 2001; 14(1):45-55.
148. Calado DP, Sasaki Y, Godinho SA, Pellerin A, Kochert K, Sleckman BP, de Alboran IM, Janz M, Rodig S and Rajewsky K. The cell-cycle regulator c-Myc is essential for the formation and maintenance of germinal centers. Nat Immunol. 2012; 13(11):1092-1100.
149. Nikitin PA, Price AM, McFadden K, Yan CM and Luftig MA. Mitogen-induced B-cell proliferation activates Chk2-dependent G1/S cell cycle arrest. PLoS One. 2014; 9(1):e87299.
150. Goodnow CC, Vinuesa CG, Randall KL, Mackay F and Brink R. Control systems and decision making for antibody production. Nat Immunol. 2010; 11(8):681-688.

Attachments

1) Activation of ATR-Chk1 pathway facilitates EBV-mediated transformation of primary tonsillar B-cells

Vanessa Mordasini, Seigo Ueda, Roberta Aslandogmus, Christoph Berger, Claudine Gysin, Daniela Hühn, Alessandro A. Sartori, Michele Bernasconi, and David Nadal

Manuscript published

2) IRAK4 is essential for TLR9-induced suppression of Epstein-Barr virus *BZLF1* transcription in Akata Burkitt's lymphoma cells

Marc Jordi, Jeannine Marty, Vanessa Mordasini, Anna Lünemann, Scott McComb, Michele Bernasconi, David Nadal

Manuscript submitted for publication

www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 4), pp: 6461-6474

Research Paper

Activation of ATR-Chk1 pathway facilitates EBV-mediated transformation of primary tonsillar B-cells

Vanessa Mordasini[1], Seigo Ueda[1,2], Roberta Aslandogmus[1], Christoph Berger[3], Claudine Gysin[4], Daniela Hühn[5], Alessandro A. Sartori[5], Michele Bernasconi[1,*], David Nadal[1,*]

[1]Experimental Infectious Diseases and Cancer Research, University Children's Hospital of Zürich, Zürich, Switzerland

[2]Department of Otolaryngology-Head & Neck Surgery, Asahikawa Medical University, Asahikawa, Japan

[3]Division of Infectious Diseases and Hospital Epidemiology, University Children's Hospital of Zürich, Zurich, Switzerland

[4]Division of Otolaryngology, University Children's Hospital of Zürich, Zurich, Switzerland

[5]Institute of Molecular Cancer Research, University of Zürich, Zurich, Switzerland

[*]These authors contributed equally to this work

Correspondence to: David Nadal, **email:** david.nadal@kispi.uzh.ch

Keywords: *EBV, DDR, ATR, Chk1, hyperproliferation*

Received: *October 04, 2016* ***Accepted:*** *December 18, 2016* ***Published:*** *December 23, 2016*

ABSTRACT

Primary infection of the immunocompromised host with the oncovirus Epstein-Barr virus (EBV) that targets mainly B-cells is associated with an increased risk for EBV-associated tumors. The early events subsequent to primary infection with potential for B-cell transformation are poorly studied. Here, we modeled *in vitro* the primary infection by using B-cells isolated from tonsils, the portal of entry of EBV, since species specificity of EBV hampers modeling in experimental animals. Increasing evidence indicates that the host DNA damage response (DDR) can influence and be influenced by EBV infection. Thus, we inoculated tonsillar B-cells (TBCs) with EBV-B95.8 and investigated cell proliferation and the DDR during the first 96 hours thereafter. We identified for the first time that EBV infection of TBCs induces a period of hyperproliferation 48-96 hours post infection characterized by the activation of ataxia telangiectasia and Rad3-releated (ATR) and checkpoint kinase-1 (Chk1). Whereas inhibition of Chk1 did not affect B-cell transformation, the specific inhibition of ATR robustly decreased the transformation efficiency of EBV. Our results suggest that activation of ATR is key for EBV-induced B-cell transformation. Thus, targeting the interaction between ATR/Chk1 and EBV could offer new options for the treatment of EBV-associated malignancies.

INTRODUCTION

Environmental and endogenous challenges constantly endanger the integrity of the genetic information by inducing DNA damage. Eukaryotic cells have developed several powerful mechanisms to repair DNA damage, thereby preventing genomic instability and avoiding tumorigenesis [1]. Depending on the type of lesion, specific DNA damage response (DDR) protein kinases are activated. In general, single-stranded regions of DNA coated by the replication protein A (RPA) promote activation of ataxia telangiectasia and Rad3-related (ATR) [2, 3], whereas DNA double-strand breaks (DSBs) recognized by the MRE11-RAD50-NBS1 (MRN) complex primarily activate ataxia telangiectasia mutated (ATM) [4, 5]. Downstream of ATM and ATR, phosphorylation events are initiated to activate a huge variety of transducer and effector proteins, such as the serine-threonine checkpoint effector kinases Chk1 and Chk2 [6]. The latter will, in turn, phosphorylate additional effectors proteins, which can induce activation of specific DNA damage checkpoints leading to cell cycle arrest, thereby allowing time for the repair of damaged DNA. This prevents DNA replication or cell division in the presence of damaged genomic material and the spreading of possible deleterious

mutations. Should DNA repair not be possible to prevent a catastrophic level of genomic instability, the DDR can activate different downstream pathways that result into programmed cell death or cell senescence and eliminate the potential threats.

There is increasing evidence that the oncogenic gammaherpesvirus Epstein-Barr virus (EBV), which mainly targets B-cells and establishes chronic B lymphocellular infection, modulates the activity of the cellular DDR [7–10]. In particular, Nikitin and colleagues showed that activation of the ATM/Chk2 signaling pathway after *in vitro* EBV infection of B-cells is critical for the suppression of EBV-mediated B-cell transformation and can act as an innate tumor suppression pathway [11].

EBV infects more than 95% of the world's population [12]. The nasopharyngeal lymphoid system, including tonsils, is the portal of entry for EBV that targets and resides in B-cells for the life-time of the host. Thus, following EBV exposure, tonsillar B-cells (TBCs) are most likely the first B-cells targeted by the virus. After primary infection, EBV establishes reversible latency in B-cells and persists there mostly as a long lasting asymptomatic infection in a rather stable pool of resting memory B-cells that circulate in the peripheral blood [13, 14]. Lytic reactivation in the nasopharynx allows host-to-host transmission of EBV via saliva to susceptible hosts [15]. Although EBV infection is harmless in the vast majority of cases, latent EBV infection is strongly associated with tumors such as endemic Burkitt's lymphoma, Hodgkin lymphoma, and post-transplant lymphoproliferative disease (PTLD) [16]. Indeed, *in vitro* infection of B-cells with EBV results in expression of all EBV's latency genes and eventually in cell transformation with the outgrowth of lymphoblastoid cell lines, thus reflecting EBV's oncogenic potential [17–19].

Primary EBV infection induces both a humoral and a cell-mediated immune response [20]. The humoral response mainly limits the spreading of the infectious virus particles blocking their binding to the cellular surface receptors [20, 21]. Cytotoxic T lymphocytes (CTL)s target and kill EBV-infected B-cells, thereby playing a key role in limiting their propagation. Immunocompromised individuals lacking a fully functional immune response, such as HIV-infected patients or organ transplant recipients, are at high risk of developing EBV-related B-cell lymphoma. Even so, the iatrogenic immunosuppression necessary to avoid graft rejection in solid organ transplantation leads to PTLD development in only up to 10% of the patients [22], suggesting that in addition to the adaptive cellular immune responses other mechanisms may play an important role in preventing the development of EBV-associated B-cell malignancies. One such additional protective mechanism could be the nature of the activated DDR since it has been identified as a major component of the underlying tumor suppressor mechanism upon EBV infection [11].

Here, we investigated the DDR in TBCs in response to EBV inoculation. We chose TBCs since they are likely the first host B-cells to be confronted with the virus upon primary infection with EBV which, in turn, is associated with the highest risk for PTLD in transplant recipients [13].

RESULTS

Tonsillar B-cells hyperproliferate in the first 96 hours post EBV inoculation

Peripheral blood B-cells inoculated with EBV *in vitro* manifest subsequently a phase of hyperproliferation of 96 hours [11]. Since palatine tonsils are located at the portal of entry for EBV, TBCs are most likely the first B-cells to be targeted by EBV following primary infection of the host, i.e., in the absence of adaptive specific immunity. Given that TBCs and B-cells circulating in the peripheral blood may phenotypically and functionally differ [23], we interrogated whether EBV inoculation *in vitro* also induces hyperproliferation of isolated TBCs. To this end, we inoculated purified CD19+ TBCs with EBV-B95.8, produced in the marmoset B95.8 cell line exposed to 12-O-tetradecanoylphorbol-13-acetate (TPA), at a multiplicity of infection (MOI) of 8, and stained the TBCs with the proliferation dye CFSE. We monitored the proliferation of TBCs at 48, 72, 96, 120, and 144 hours post inoculation (pi) using flow cytometry (Figure 1A). Non-inoculated purified CD19+ TBCs were grown for 120 hours and used as negative control (mock inoculation). EBV-inoculated CD19+ TBCs started to proliferate after 48 hours and divided more than once between 48 and 72 hours, as indicated by the number of peaks detected by CFSE staining. In addition, EBV-inoculated CD19+ TBCs proliferated faster between 48 and 96 hours than at later time points as indicated by a rapid decrease in CFSE staining (Figure 1A). In contrast, CD19+ TBCs did not proliferate within the first 120 hours post mock-inoculation. Thus, EBV induces proliferation of CD19+ TBCs *in vitro* that peaks between 48 and 96 hours post inoculation.

Next, we wanted to investigate whether proliferating TBCs undergo a phase of hyperproliferation similar to the one reported for peripheral blood B-cells [11]. Thus, we further analyzed data from the CFSE staining experiments (Figure 1A) as suggested by Hawkins *et al.* [24]. Briefly, we calculated the mean division number (MDN) fitting a Gaussian distribution to the precursor-normalized number of cells in each division. For each TBC donor, we plotted the MDN *versus* the analyzed time point (Figure 1B). The slope of this function represents the number of divisions per hour and inversely correlates with the proliferation rate. The proliferation rate of TBCs decreased over time (Figure 1B), indicating that cells hyperproliferated between 48 and 96 hours after EBV inoculation but not thereafter.

Taken together, these data demonstrate that CD19+ TBCs behave similarly to circulating peripheral blood B-cells, undergoing a phase of hyperproliferation during the first 4 days (96 hours) post *in vitro* EBV inoculation.

EBV induces activation of the ATR/Chk1 pathway in tonsillar B-cells

Cellular hyperproliferation implicates augmented frequency of DNA replication, which can lead to high levels of DNA damage resulting in so called replication stress [25]. ATR and its downstream effector kinase Chk1 are both key players in the cellular response to DNA replication stress [26, 27].

To investigate if ATR and Chk1, as to be expected during hyperproliferation, are activated in TBCs in the observed hyperproliferation phase following EBV inoculation, we measured their protein expression and phosphorylation levels in purified CD19+ TBCs at 24, 48, 72, and 96 hours post EBV inoculation by western blotting (Figure 2A). As positive and negative controls, we used EBV-induced lymphoblastoid cell lines (LCLs) treated with etoposide, a DNA topoisomerase II inhibitor known to induce DSBs and SSBs and mock-inoculated purified CD19+ TBCs harvested 24 hours post-isolation, respectively. We detected a gradual increase in the phosphorylation of ATR at serine 428 (pATR) and of Chk1 at serine 345 (pChk1) during the course post EBV inoculation, with a robust signal for pChk1 at 72 hours (Figure 2A and 2B). This period coincided with the previously observed hyperproliferation phase (Figure 1B), suggesting that indeed EBV-inoculated TBCs experience replication stress during the hyperproliferation phase observed between 48 to 96 hours post EBV inoculation. Remarkably, we also observed a strong increase in total ATR and Chk1 protein levels already at 24 hours after EBV inoculation (Figure 2A). Increased ATR and Chk1 protein levels can be triggered either by increased *de novo* transcription and translation of the protein, by decreased protein degradation or by increased protein stability [28, 29]. Furthermore, analysis of both ATR and Chk1 mRNA expression levels by real-time quantitative PCR (Figure 2C) showed an increased mRNA expression for both ATR and Chk1 starting already at 24 hours post EBV inoculation. This suggested that the increase in total ATR and Chk1 protein levels was rather due to increased *de novo* transcription and translation rather than decreased protein degradation.

Kaneko *et al.* [29] showed that expression of Chk1 is increased specifically at the S to M phase of the cell cycle at both the RNA and protein levels, whereas ATR seems to be expressed throughout the cell cycle at constant levels [30]. Indeed, in our analysis of the cell cycle of EBV inoculated TBCs by PI staining we found an increase in the S-G2/M population starting at 72 hours post EBV inoculation (Figure 2D and Supplementary Figure S1A). This explained, at least in part, why we documented such a strong increase in Chk1 expression.

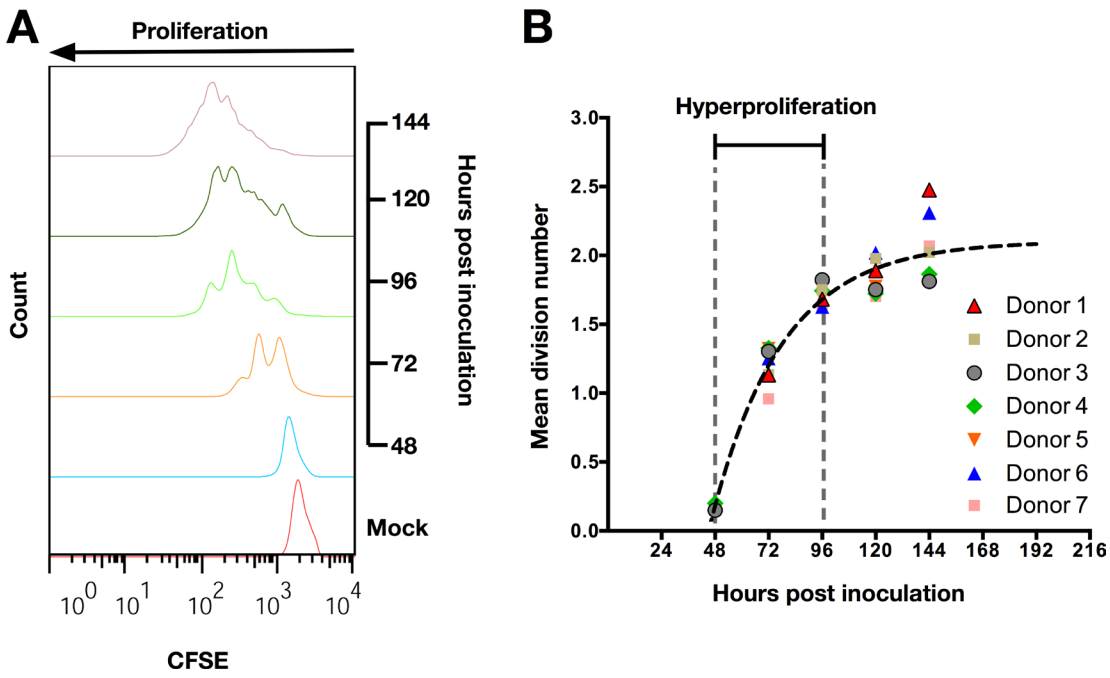


Figure 1: EBV induces hyperproliferation of tonsillar B-cells (TBCs) within 72 hours post inoculation. (**A**) Representative data (Donor 7 from Figure 1B) of CFSE proliferation profile of TBCs at sequential days post EBV-B95.8 inoculation. Non-inoculated CD19+ B-cells harvested 120 hours post isolation (Mock) were used as negative control. (**B**) Mean division number of TBCs at sequential days post EBV-B95.8 inoculation. The function depicted as a black dashed line represents the average mean division number (MDN) for each time point measured. . Results shown are from TBCs from 7 donors.

Collectively, the observed chronological correlation between hyperproliferation and activation of the ATR/Chk1 pathway together with an accumulation of cells in S-G2/M phase further strengthens the notion that TBCs experience a phase of replication stress early after EBV inoculation in this system with the absence of specific adaptive immunity.

EBV does not induce activation of the ATM/Chk2 pathway in tonsillar B-cells

Besides ATR/Chk1, a second kinase signaling cascade consists of the ATM and Chk2 protein kinases, which are mainly activated by DSBs rather than replication stress [31]. Furthermore, activation of Chk2 has been reported to occur following EBV infection of peripheral blood B-cells *in vitro* [11]. Therefore, we analyzed activation of ATM and Chk2 in the identical samples shown in Figure 2. Similar to ATR and Chk1, we also noted a robust increase in total ATM and Chk2 protein levels 24 hours after EBV inoculation (Figure 3A), confirmed by analysis of both ATM and Chk2 mRNA expression levels by real-time quantitative PCR (Figure 3D). In addition, we also detected a low activation of pATM and pCHk2 especially 24 hours post EBV inoculation (Figure 3A). However, the activation was limited compared to the etoposide control (Figure 3B) and did not affect activation of the ATM substrate KAP1 (pKAP1 at serine 824) that controls DSB repair in heterochromatin [32] (Figure 3C). Interestingly, similarly to ATM and Chk2, we could also observe an increase of both total KAP1 protein levels and mRNA expression

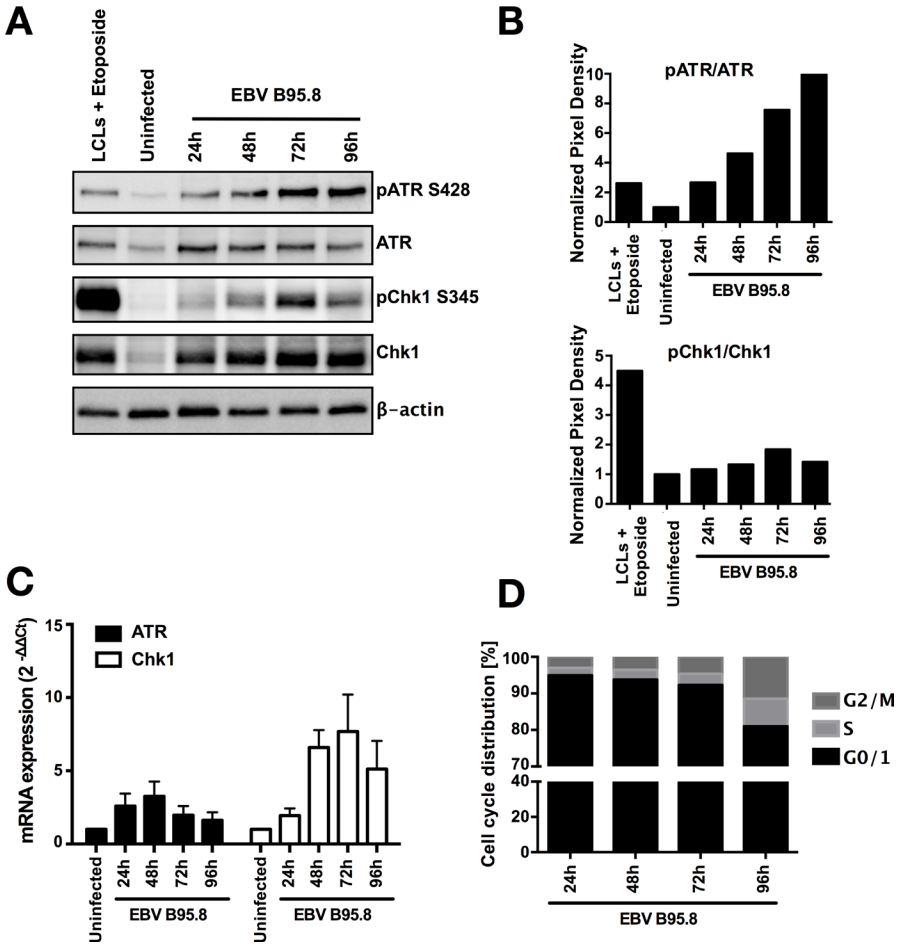


Figure 2: EBV inoculation elicits ATR/Chk1-mediated DDR activation in tonsillar B-cells (TBCs). TBCs where inoculated with EBV-B95.8 (MOI 8). (**A**) Cells were harvested at the indicated time points and expression of total ATR and Chk1 and pATR S428 or pChk1 S345 was analyzed by western blotting. Non-inoculated TBCs at 24 hours post isolation were used as negative control. Lymphoblastoid cell lines (LCLs) treated with Etoposide for 6 hours served as positive control. The results shown are representative of 3 independent experiments (**B**) ATR and Chk1 phosphorylation was quantified by densitometric analysis. Data are represented as ratio of phosphorylated-to-total. Quantification was performed using the software imageJ 1.49t. The results shown are representative of 3 independent experiments (**C**) ATR and Chk1 mRNA expression was determined in EBV-inoculated B-cells at the indicated time points by RTqPCR relative to 18S RNA and shown as fold change relative to non-inoculated cells. Data are presented as mean ± SEM of 4 donors. (**D**) Cell population in G1, S, G2/M at different time points post EBV-inoculation were quantified by flow cytometry based cell cycle analysis measured by labeling the fixed cells with propidium iodide (PI). Results shown are from 3 donors and are presented as mean.

levels (Figure 3C and 3D) post EBV inoculation. The absence of KAP1 activation strongly supports the absence of ATM/CHk2 pathway activation in TBCs following EBV inoculation. Nevertheless, the observed limited activation of ATM and Chk2 could explain previous reports on the activation of the ATM pathway by EBV.

Taken together, these results document that the ATM/Chk2 pathway is not activated in purified CD19+ TBCs inoculated with EBV *in vitro*.

EBV activates the ATR/Chk1 pathway also in peripheral blood B-cells

Given that EBV infection of peripheral blood B-cells *in vitro* was reported to activate ATM and Chk2 but the activation of ATR and Chk1 was not referred to [11], we interrogated whether activation of the ATR/Chk1 dependent pathway is dependent or independent of the infected B-cell origin, i.e., tonsil versus peripheral blood. Thus, we purified CD19+ peripheral B-cells and inoculated them with EBV-B95.8 similarly as isolated CD19+ TBCs. We then monitored the protein expression and phosphorylation levels of ATR, Chk1, ATM and Chk2 at 24, 48, 72, and 96 hours post EBV inoculation by western blotting (Figure 4A and 4B). Similarly to TBCs (Figures 2A and 3A), we observed activation of the ATR/Chk1 dependent pathway in peripheral blood B-cells following EBV inoculation, indicated by a reproducible increase of the phosphorylation for both ATR and Chk1 (Figure 4A and 4C). This suggested that activation of ATR and Chk1 following EBV inoculation *in vitro* is not dependent on the origin of the B-cells.

Surprisingly, in contrast to what was reported we did not detect activation of the ATM/Chk2 dependent pathway in peripheral blood B-cells following EBV inoculation (Figure 4B and 4C) except for a minimal increase of the ATM total-to-phosphorylation ratio after EBV infection similar to what we observed in TBCs

(Figure 3B). This suggested that distinct experimental procedures in the reported study [11] versus our study were likely responsible for the contrasting results in terms of ATM/Chk2 activation in peripheral blood B-cells.

Activation of ATR/Chk1 is not EBV-strain dependent

Next, we interrogated whether activation of the ATR/Chk1 signaling pathway in CD19+ TBCs is dependent on the strain of EBV used for inoculation. To this end, we monitored Chk1 and Chk2 phosphorylation following inoculation of TBCs with two distinct EBV strains. We inoculated purified CD19+ TBCs with EBV-B95.8 or with EBV-GFP produced in HEK-293 cells. Consistent with our previous results, we observed that pChk1, but not pChk2, was induced at 72 hours post-inoculation either with EBV-B95.8 or with EBV-GFP (Figure 5A).

Thus, activation of the ATR/Chk1 pathway in TBCs following inoculation is not an EBV strain-dependent phenomenon, at least not with the two strains tested.

Hyperproliferation of CD19$^+$ TBCs induced by CD40L activates Chk1

Since ATR/Chk1 activation does not seem to be EBV-strain specific, we asked whether the observed activation of ATR/Chk1 was EBV-dependent at all. Thus, we wondered whether CD40L, a B-cell stimulant with similar effects as EBV regarding B-cell survival and hyperproliferation [33], did also activate ATR/Chk1. To answer this question, we used CD40L in combination with IL-4 to stimulate proliferation of TBCs similar to the one observed in EBV-infected B-cells but in an EBV-independent fashion. We treated purified CD19+ TBCs with CD40L in combination with IL-4 for 72 hours and measured the levels of, pATR, pChk1, pATM and pChk2 by western blotting. Indeed, inoculation of isolated CD19+ TBCs with CD40L resulted in activation of ATR and Chk1 at 72 hours post stimulation (Figure 5B).

Thus, considering that peripheral blood B-cells undergo a phase of hyperproliferation at 72 hours post CD40L stimulation [33], activation of the ATR/Chk1-dependent pathway seems not to be a strictly EBV-related phenomenon in B-cells but rather seems to be linked to the hyperproliferation of the B-cells.

To verify if activation of the ATR/Chk1 pathway is dependent from productive EBV infection or rather form EBV or cellular components that might have co-purified with the virus, we monitored ATR, Chk1, ATM and Chk2 following inoculation of purified CD19+ TBCs with EBV-B95.8 or UV-inactivated EBV-B95.8. Contrasting with ATR/Chk1 activation following inoculation with live EBV-B95.8, UV-inactivated EBV-B95.8 did not show any phosphorylation of ATR and Chk1 (Figure 5C). This demonstrated that the UV-inactivated EBV does not activate ATR/Chk1-dependent pathway as live EBV, excluding that other elements present in the viral supernatant are responsible for Chk1 activation. Thus, TBCs need to experience EBV infection rather than mere contact with EBV or its elements to manifest ATR/Chk1 activation during hyperproliferation following EBV inoculation (Figure 1A).

Taken together, these results confirm that hyperproliferation is sufficient to induce activation of ATR/Chk1 in purified CD19+ B-cells This, in turn, may indicate that B-cells counteract their vulnerable phase of primary EBV infection mirrored by hyperproliferation by activating the ATR/Chk1-related pathway.

ATR activation increases EBV-mediated transformation of TBCs

EBV has the ability to transform B-cells *in vitro* [13, 34]. Thus, we were interested to know whether activation of the specific ATR/Chk1-mediated pathway within 96 hours post EBV inoculation *in vitro* has any consequences for the subsequent transformation of TBCs, since the risk for PTLD in transplant recipients is highest following primary EBV infection but is also present in EBV carriers [22].

More specifically, we mimicked the *in vivo* situation of primary EBV B-cell infection in iatrogenically immunosuppressed transplant recipients by using tonsillar mononuclear cells (TMCs), rather than purified CD19+ TBCs, from EBV seronegative donors, to mimic

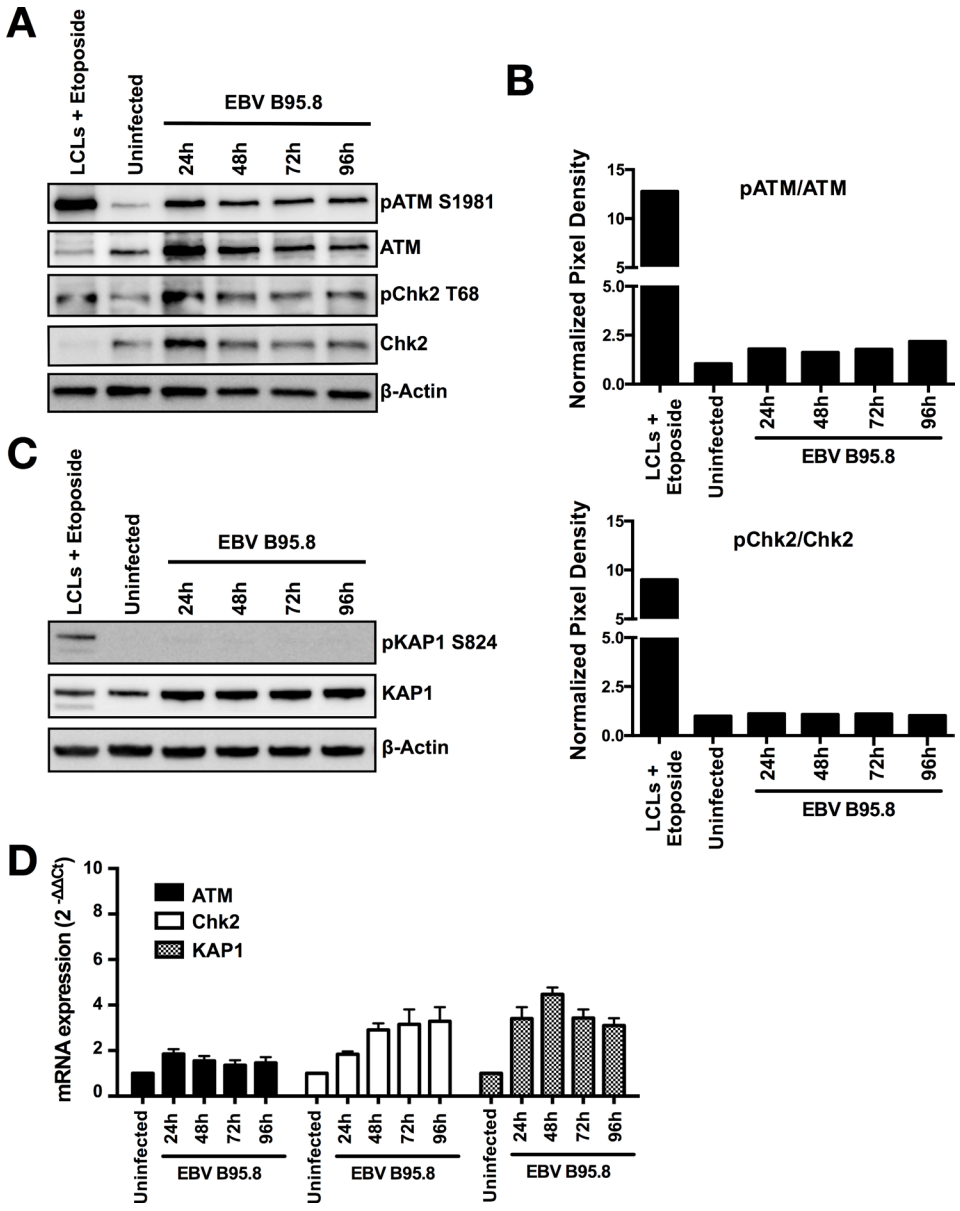


Figure 3: EBV inoculation does not elicit ATM/Chk2-mediated DDR activation in tonsillar B-cells (TBCs). TBCs where inoculated with EBV-B95.8 (MOI 8). Non-inoculated TBCs at 24 hours post isolation were used as negative control (uninfected). Lymphoblastoid cell lines (LCLs) treated with Etoposide for 6 hours served as positive control. (**A**) Cells were harvested at the indicated time points, and expression of total ATM or Chk2 and pATM S1981 or pChk2 T68 was analyzed by western blotting. (**B**) ATM and Chk2 phosphorylation and total protein amount were quantified by densitometric analysis. Data are represented as ratio of phosphorylated-to-total. Quantification was performed using the software imageJ 1.49t. (**C**) Total KAP1 and pKAP1 S824 was analyzed by western blotting. The results shown are representative of 3 independent experiments. (**D**) ATM, Chk2 and KAP1 mRNA expression was determined in EBV-inoculated B-cells at the indicated time points by RTqPCR relative to 18 S RNA and shown as fold change relative to non-inoculated cells. Data are presented as mean ± SEM of 4 donors.

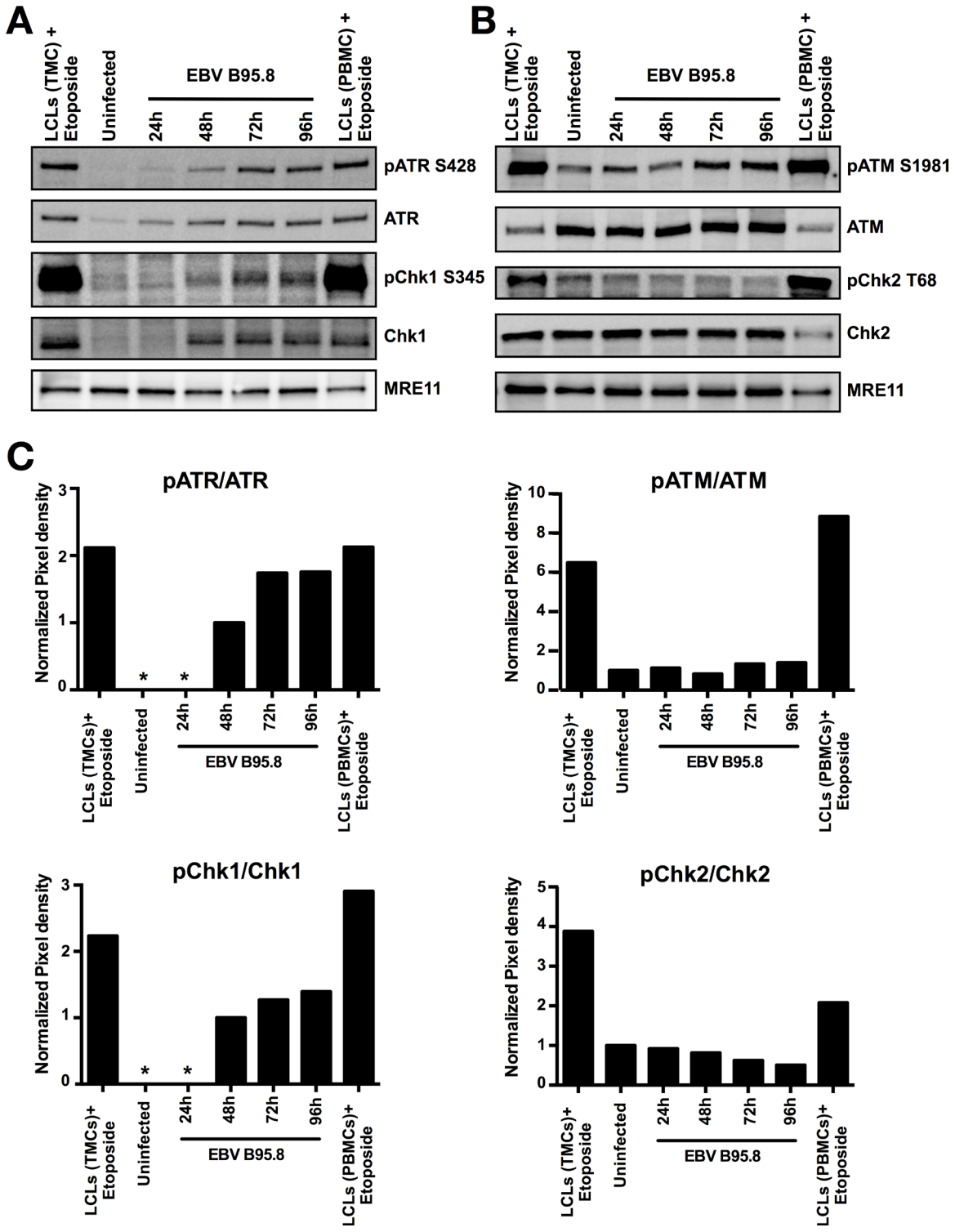


Figure 4: EBV inoculation elicits ATR/Chk1-mediated pathway activation but not ATM/Chk2 in peripheral B-cells (PBCs). Peripheral B-cells were inoculated with EBV-B95.8 (MOI 8). Non-inoculated peripheral B-cells at 24 hours post isolation served as negative control (Uninfected), Lymphoblastoid cell lines (LCLs) derived from TMCs or PBMCs treated with Etoposide for 1 hours served as positive control. Detection of MRE11 served as loading control. (**A**) Cells were harvested at the indicated time points and expression of total ATR or Chk1 and pATR S428 or pChk1 S345 was analyzed by western blotting. (**B**) Cells were harvested at the indicated time points, and expression of total ATM or Chk2 and pATM S1981 or pChk2 T68 was analyzed by western blotting. The results shown are representative of 3 independent experiments. (**C**) ATR, ATM, Chk1 and Chk2 phosphorylation and total protein amount were quantified by densitometric analysis. Data are represented as ratio of phosphorylated-to-total. Quantification was performed using the software imageJ 1.49t. * = Not detectable.

primary host EBV infection. We also used TMCs from EBV seropositive donors treated with cyclosporine A to suppress T cell function, to mimic primary B-cell infection in immunosuppressed EBV carriers. We used two distinct inhibitors: VE-821, specific for ATR (ATRi) [35]; and CHIR-124, specific for Chk1 (Chk1i) [36]. Specificity of VE-821 and CHIR-124 were assessed by monitoring the phosphorylation levels of Chk1 at S345 and S296 [37] respectively (Supplementary Figure S2A and S2B). To avoid artifacts that may result from inhibitors toxicity we also assessed the cell viability after treatment with VE-821 and CHIR-124 at different concentration (Supplementary Figure S2C).

TMCs were treated with ATRi or Chk1i from the start of the inoculation (day 0 pi) or whilst in the hyperproliferation phase (4 days pi). Specifically, we inoculated TMCs with decreasing MOIs of EBV according to a 3-fold serial dilution. At 5 weeks pi, we determined the EBV transformation efficiency by counting the number of wells positive for B-cells outgrowth. The percentage of transformed wells was plotted relative to the transforming units (TU) of EBV used per well. TUs per well were calculated assuming that 62.5% of wells were transformed by 1 TU. Transformation efficiency was assessed comparing how many TUs were needed to transform 62.5% of wells. ATR inhibition at day 0 or

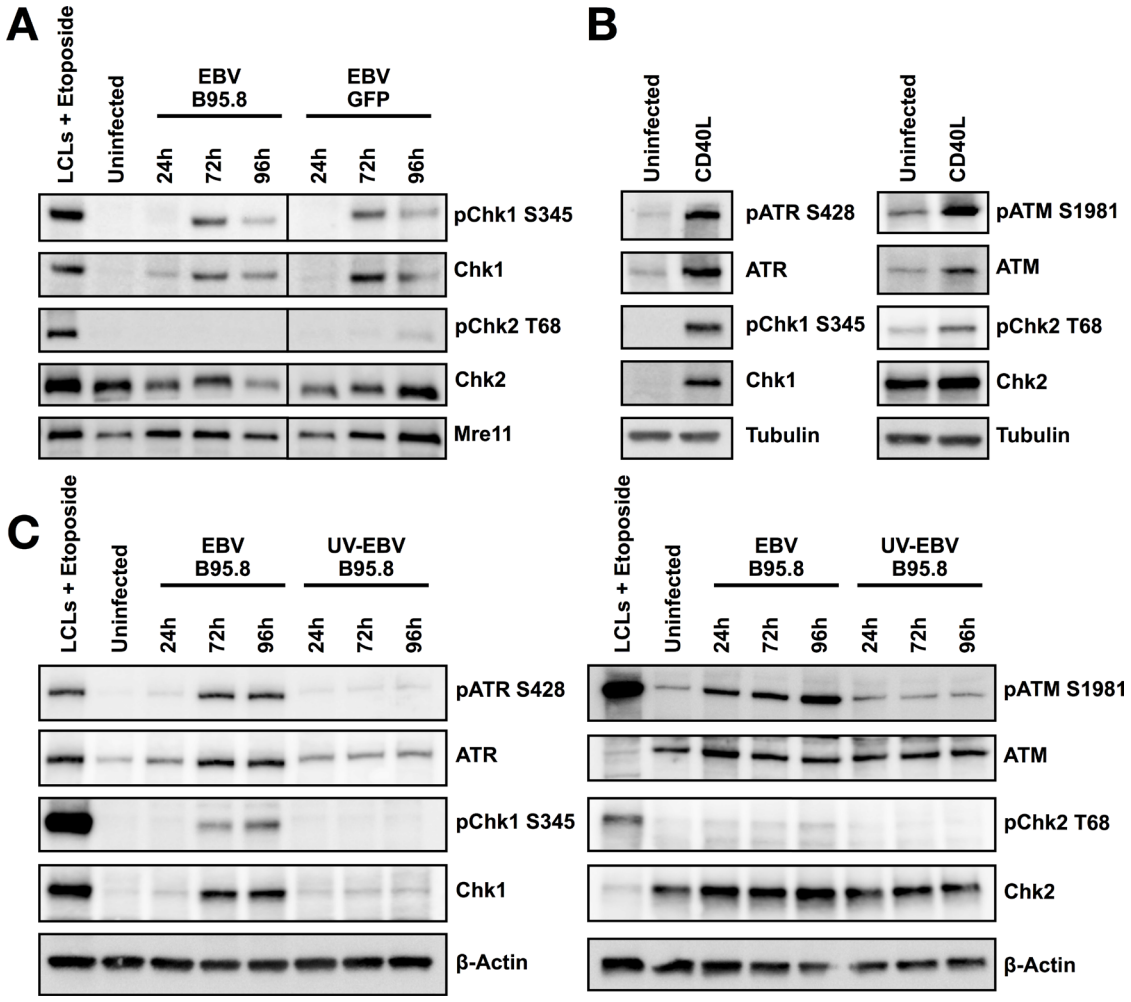


Figure 5: Hyperproliferation of CD19+ TBCs induced by CD40L activates Chk1 but not inoculation with UV inactivated EBV-B95.8 activates Chk1. (**A**) TBCs were inoculated with EBV-B95.8 or EBV-GFP (MOI 8). Cells were harvested at the indicated time points and expression of total Chk1 and pChk1 S345 or total Chk2 and pChk2 T68 was analyzed by western blot. Non-inoculated TBCs at 24 hours post isolation served as negative control, lymphoblastoid cell lines (LCLs) treated with Etoposide for 1 hour served as positive control. Detection of MRE11 served as loading control. The results shown are representative of 3 independent experiments. (**B**) TBCs where treated with 5 ng/mL CD40L, 20 ng/mL Il-4 and 200 ng/mL HA-antibody for 72 h. Expression of pATR, pChk1, pATM or pChk2 was analyzed by western blot. Non-inoculated TBCs at 24 hours post isolation served as negative control. Tubulin served as loading control (**C**) TBCs were inoculated with EBV-B95.8 or UV-EBV-B95.8 (MOI 8). Cells were harvested at the indicated time points and expression of pATR, pChk1, pATM or pChk2 was analyzed by western blotting. Non-inoculated TBCs at 24 hours post isolation served as negative control, lymphoblastoid cell lines (LCLs) treated with Etoposide for 6 hours served as positive control. Detection of ß-Actin served as loading control. The results shown are representative of 3 independent experiments.

4 pi resulted in a decrease in transformation efficiency compared to the vehicle control DMSO of ~ 1.5 and to ~ 2.3 fold respectively (Figure 6A). By contrast, inhibition of Chk1 did not affect EBV transformation efficiency neither at day 0 pi nor 4 pi compared to the DMSO control (Figure 6B).

In conclusion, activation of ATR during the first 4 days pi, i.e. during the phase of hyperproliferation post EBV inoculation, seemed to be a key factor, which increased EBV transformation efficiency *in vitro*. Thus, factors impacting on this phase of vulnerability are likely to determine whether eventually EBV-induced transformation or PTLD occur.

DISCUSSION

Here, we investigated the role of the DNA damage response (DDR) pathways in controlling proliferation and transformation of TBCs during EBV primary infection using an *in vitro* system to model primary EBV infection of the EBV naïve host without and with immunosuppression, the latter close to primary B-cell infection in the immunosuppressed transplant recipient. We found that following inoculation with EBV (i) TBCs undergo a phase of hyperproliferation; (ii) in that phase TBCs express pATR and pChk1, suggesting that these abnormally proliferating cells experience replication stress; and (iii) ATR inhibition in TBCs decreases EBV's transformation efficiency. Our results unprecedentedly show how a cellular mechanism, aimed at protecting DNA from damage induced by abnormal cell proliferation, opens a vulnerable phase to transformation for the target cells following primary EBV infection of the host or of a B-cell.

Remarkably, analysis of TBCs proliferation revealed that B-cells enter their first round of cellular DNA replication only between 48 to 72 hours after EBV inoculation (Figure 1A). Subsequent mathematical analysis (Figure 1B) showed that TBCs undergo a phase of hyperproliferation between 48 and 96 hours post EBV inoculation. Our observation is comparable to the findings by Nikitin *et al.* [11] who described a phase of hyperproliferation approximately between 2.5 and 4.5 days after EBV infection of peripheral blood B-cells *in vitro*. During hyperproliferation, defined as an abnormally high rate of cell division, there are higher chances for the cells to drive a faulty DNA replication. This can result in high level of replication stress and subsequently in activation of

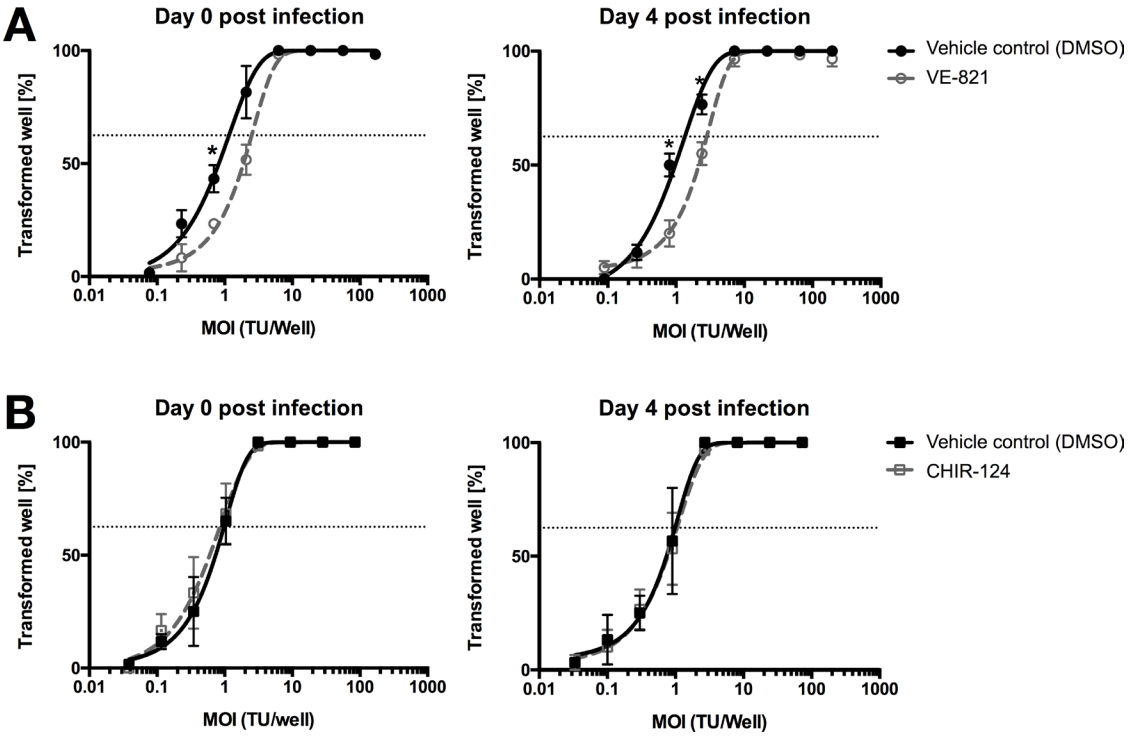


Figure 6: ATR inhibitor reduces EBV's B-cell transformation efficiency. (**A**) Quantification of B-cells outgrowth following tonsillar mononuclear cells (TMCs) infection with EBV-95.8 (3 fold serial dilution) in presence of vehicle control (0.01% of DMSO - black line) or 1 μM VE-821 ATR inhibitor (dottet-grey line) added at day 0 or 4 post EBV inoculation. (**B**) Quantification of B-cells outgrowth following TMCs in presence of vehicle control (0.01% DMSO - black line) or 10 nM of CHIR-124 Chk1 inhibitor (dotted-grey line) added at day 0 or 4 post EBV inoculation. The number of transformed wells is plotted relative to the transforming units (TU) of EBV-B95.8 per well. Results shown are from 3 donors and are presented as mean ± SEM. For both figure (A) and (B) two EBV positive and one EBV negative donors were used. Dotted lines represent 62.5% positive wells. Based on a Poisson distribution this percentage represents the probability of B-cells outgrowth knowing that a viral particle is present in every well. *= $p < 0.05$ (Unpaired *t*-Test).

a strong and specific DDR [25]. Human Herpesvirus 6 has been shown to induce activation of Chk1 72 hours pi [38], similarly to EBV (human herpes virus 4) as documented here. Thus, activation of an ATR/Chk1-dependent pathway in EBV-inoculated TBCs, as shown here for the first time, might be an alternative mechanism of defense activated by target cells in the absence of a functional CTLs response limiting outgrowth and transformation of EBV-infected B-cells.

Of note, B-cells exhibited activation of ATR and Chk1 as indicated by phosphorylation of S428-ATR and S345-Chk1 (Figures 2A, 4A), which strictly coincided with the hyperproliferation phase following exposure to EBV. It has been shown that in response to CD40L/IL4 stimulation B-cells undergo an initial burst of hyperproliferation similar to what has been described for EBV infection [33]. Since we observed similar activation of ATR and Chk1 in TBCs following stimulation with CD40L/IL-4 (Figure 5B) the process is not EBV-specific but rather a consequence of B-cell activation leading to replication stress. Nevertheless, the absence of ATR/Chk1 activation when inoculating TBCs with UV-inactivated EBV (Figure 5C) strongly implies that EBV needs to actively infect the target cell to induce ATR/Chk1 activation.

By contrast, TBCs did not exhibit detectable activation of ATM and Chk2 in any of our experiments (Figures 3A and 4A and 5A), suggesting absence of DSB leading to the activation of the required ATM/Chk2-mediated DDR. Our results are at variance to those of Nikitin *et al.* [11] who observed activation of the ATM/Chk2-mediated DDR in peripheral blood B-cells following EBV infection *in vitro*. One possible explanation could be the distinct EBV sources used in the experiments. We exposed TBCs to a second EBV strain (EBV-GFP) and obtained the similar DDR pattern as we obtained with EBV-B95.8, suggesting that distinct EBV strains are not a very likely explanation for the results at variance. An alternative explanation could be the distinct sources of B-cells (TBCs versus peripheral blood B-cells). We executed the assessment of DDRs also with peripheral blood B-cells, but the results did not differ from those obtained with TBCs (Figure 4A). Thus, although the reason for the results at variance remains elusive, distinct experimental conditions but not the virus strains or the B-cell tissue origin are the most likely cause.

A most remarkable and surprising finding was the reduced EBV *in vitro* transformation efficiency observed after ATR inhibition (Figure 6A), indicated by the increase in the number of TUs necessary to transform 62.5% of cells after ATR inhibition. No such changes were detected after Chk1 inhibition (Figure 6B). Interestingly, Nikitin and colleagues [11] described that inhibition of ATM and Chk2 exhibited an opposite effect on EBV transformation efficiency in peripheral blood B-cells, i.e., an increased transformation efficiency of EBV. This suggested that activation of this specific DDR pathway works as an alternative tumor suppressive pathway that regulates B-cell transformation. Thus, distinct activation of specific DDRs seems to have diverse effects on EBV-induced B-cell transformation *in vitro*. This, in turn, may imply that depending on the activated DDR upon EBV exposure in the absence of adaptive immune control of EBV-infected B-cells distinct outcomes of EBV-mediated B-cell transformation may arise. Further exploration of the mechanisms involved may reveal potential interventional targets for patients with deficient immunity against EBV, e.g., organ transplant recipients or HIV-infected individuals.

Even though EBV plays a crucial role in the development of PTLD, *in vivo* only 10% of the transplant recipients develop EBV-related lymphoma. Thus, additional factors might be required in order to induce PTLD [22]. One could speculate that previous bacterial or viral infections might stimulate TBCs proliferation leading to replication stress and consequently activation of the ATR/Chk1-mediated DDR, increasing the probability of EBV transformation by creating an environment promoting lymphoblastoid cell transformation by EBV. Indeed, it is known that TLRs signaling, including TLR9 that bind specific common patterns on bacterial DNA (CpG motifs), can directly induce B-cell proliferation [33, 39]. Furthermore, it has been shown in our lab that triggering of TLR9 signaling in a Burkitt's lymphoma derived cell line blocks reactivation of EBV lytic cycle, reinforcing the risk of cell transformation [40]. Additional factors might also be required *in vivo*, in addition to ATR, to significantly increase the risk of B-cell transformation by EBV. Those factors might be expressed only by certain individuals and therefore explain why PTLD is not more prevalent within the immunosuppressed patients.

Taken together, our results indicate that activation of the specific ATR/Chk1-mediated DDR pathway, and more precisely of ATR, is important to enhance EBV transformation efficiency in TBCs *in vitro*. *In vivo*, the immunocompetent host is able to keep hyperproliferating B-cells under control and EBV causes only very rarely B-cell tumors. By contrast, transplantation-related or otherwise highly immunosuppressed patients are at high risk of developing EBV-related PTLD upon primary EBV infection. Without an efficient adaptive immune system able to restrict proliferation of EBV-infected B-cells, those latter would not be restricted to undergo hyperproliferation.

MATERIALS AND METHODS

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The Cantonal Ethics Committee approved the study (StV29/06). All

subjects or their caregivers provided written informed consent for the collection of samples and subsequent analyses.

Cell culture

All cells were maintained in RPMI-1640 medium (Sigma-Aldrich, Buchs, Switzerland) supplemented with 10% heat-inactivated (hi) fetal bovine serum (FBS; Life Technologies-Thermo Fisher Scientific, Reinach, Switzerland), 2 mM L-Glutamine, and 100 U/ml penicillin, and 100 µg/ml streptomycin, referred to hereafter as complete medium. All cells were cultured at 37°C in 5% CO_2 air of relative humidity > 95%.

Preparation of primary B-cells

Primary human tonsillar mononuclear cells (TMCs) were isolated from palatine tonsils obtained from pediatric patients who underwent tonsillectomy due to tonsillar hyperplasia. Tonsillar B-cells (TBCs) were prepared as described previously [23]. Briefly, tonsils were cut into small pieces with a scalpel in phosphate-buffered saline (PBS) and passed through a 70 µm-pore-size cell strainer (Falcon, Wohlen, Switzerland). Then, TMCs were purified by density gradient centrifugation with Ficoll-Paque Premium (VWR international-GE Healthcare, Dietikon, Switzerland). TBCs were isolated from TMCs using the B-cell isolation kit II according to the instructions of the manufacturer (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity of isolation was always over 95% as assessed by flow cytometry.

Peripheral B-cells (PBCs) were isolated from blood form healthy donors and prepared as following. Buffycoats were diluted 5× with phosphate-buffered saline (PBS). Then, peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation with Ficoll-Paque Premium (VWR international-GE Healthcare). PBCs were isolated from PBMCs using the B-cell isolation kit II according to the instructions of the manufacturer (Miltenyi Biotech).

Preparation of virus stock and quantification

The EBV-infected marmoset B95-8 were seeded at a density of 10^6 cells/ml and were stimulated to release virus by culture for 6–7 days in complete medium containing 50 ng/ml of 12-Otetradecanoylphorbol-13-acetate (TPA; Sigma-Aldrich)/ml. Cell suspensions were centrifuged at 1000 × g for 10 min. Supernatant was passed through a 0.45 µm-pore-size cellulose acetate filter (Sarstedt, Sevelen, Switzerland) and stored at −80°C.

Recombinant EBV-GFP was produced in 293 cells as described elsewhere [41]. Briefly, 80–90% confluent HEK293-D2089 [42] cultured in DMEM supplemented with 10% hiFBS, 1% L-Glutamine, 1% Penicillin/Streptomycin, 100 µg/ml Hygromycin B (HygroGOLD; InvivoGen, Toulouse, France) were transfected with expression plasmids encoding the EBV gene BZLF1, and BALF4 (2 µg each/10cm plate) using Metafectene (Biontex, Martinsried/Planegg, Germany). Four hours after transfection, the transfection mixture was replaced by fresh supplemented DMEM without Hygromycin B. Four days after transfection, supernatant was harvested, cleared by centrifugation at 4°C with 1.000 × g for 15 min, filtered through a 0.45µm filter and stored at −80°C.

Concentrated virus stocks were prepared by centrifugation of viral supernatant with 30.000 × rpm for 2 hours at 4°C and resuspension of the virus pellet in complete medium (1/100 of the starting volume) and store at −80°C.

Ultraviolet (UV)-inactivated EBV-B95.8 was prepared by exposing 1.5 ml of concentrated virus stock for 15 min to a source of UV germicidal light.

Infection of primary B-cells and generation of lymphoblastoid cell lines (LCLs)

TBCs were centrifuged and resuspended in complete medium at a concentration of 2 × 10^6 cells/ml. B95.8 culture supernatant prepared as described previously [23], EBV-GFP or UV-inactivated EBV-B95.8 was added to each well at an MOI of 8.

Freshly isolated TMCs or PBMCs were infected with 100 µl of B95-8 EBV and immediately treated with cyclosporin A (400 ng/ml; Sigma-Aldrich) to avoid killing of EBV-infected cells by EBV-specific T cells. The cells were then seeded on a 96-wells plate and kept in culture for at least 5 weeks before expanding and freezing them in FCS/10% DMSO at −80°C.

Proliferation assays

TBCs were labeled with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma-Aldrich) for 5 min at room temperature. The cells were then washed three times with PBS containing 5% hiFBS and resuspended in complete medium. The CFSE-labeled TBCs were inoculated with B95.8 culture supernatant and kept in culture. The cells were then harvested at indicated time points and analyzed by FACSCanto II (Becton Dickinson, Allschwil, Switzerland). The percentage of divided cells was calculated using FlowJo proliferation platform and the mean division number was calculated as reported previously [24].

Western blotting

For western blotting whole cell lysate was prepared with 1% Triton X-100 Buffer (50 mM Tris-HCL, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 50 mM NaF 10 mM b-glycerolphosphate, 5 mM SodiumPyrophosphate, 1 mM

Na_3VO_4) supplemented with CompleteMini protease inhibitor (Roche Diagnostics, Rotkreuz, Switzerland). For the detection of primary antibodies, we used horseradish peroxidase-labeled goat anti-rabbit or horse anti-mouse antibodies (CellSignaling, Danvers, MA). Detection was performed using ECL western blotting detection reagents (VWR international-GE Healthcare) or SuperSignal west femto kit (Thermo Fisher Scientific). The signal was detected using the imaging system Fujifilm LAS-3000 imager (Fujifilm, Dielsdorf, Switzerland). The densitometry analysis was performed using the Image J software.

RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. After DNaseI (DNA-free; Life Technologies - Thermo Fisher Scientific) treatment, 0.5 µg-1 µg of total RNA was used as template for reverse transcription using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Gene expression of *Chk1*, *ATR, Chk2, ATM and KAP1* was determined using pre-validated primer/probes assay (Hs00992123_m1, Hs0967506_m1, Hs00200485_m1, Hs00175892_m1 and Hs00232212_m1 respectively; Applied Biosystems - Thermo Fisher Scientific). All reactions were performed on a 7900HT real-time PCR machine ((Applied Biosystems - Thermo Fisher Scientific) with TaqMan Gene Expression Master Mix (Thermo Fisher Scientific). The relative gene expression was calculated for each gene of interest by using a $\Delta\Delta C_t$ method, where C_t values were normalized to the housekeeping genes 18s RNA.

Cell cycle analysis by flow cytometry

TBCs inoculated with B95.8 were harvested at the indicated time point and washed with PBS. The cells were then fixed in 70% ethanol and kept at 4°C until staining. After fixation the cells were washed wit PBS and stained with a propidium iodide (PI)-RNAseA solution (25 µg/ml-0.1 mg/ml) for 30 min at 37°C. Stained samples were analyzed by FACS Canto II (Becton Dickinson, Allschwil, Switzerland). The results analysis was performed using the FlowJo software.

Cell viability assay

Viability of LCLs after VE-821 or CHIR-124 treatments was assayed using WST-1 (water soluble tetrazolium-1) salt as a substrate, as described in the protocol supplied with the kit (Roche Diagnostics, Rotkreuz, Switzerland). The absorbance measured using a microplate reader (Bio-TEK instruments, Luzern, Switzerland) at 450 nm. Cell viability was calculated by $A_{treatment}/A_{control} \times 100$ (A represents the absorbance recorded at 450 nm).

Transformation assay

Infection of TMCs by EBV-B95.8 was performed in presence 400 ng/ml of cyclosporine A (Sigma Aldrich), 0.01% DMSO, 1µM ATRi VE-821 (Selleckchem, Huston, TX) or 10 nM Chk1i CHIR-124 (Selleckchem) added at different times post infection (pi). TMCs were plated in 24 wells of a 96 well plate and infected with different EBV concentration (MOI from 30 to 0.014, 3-fold serial dilution). The percentages of wells positive for B-cell transformation at 5 weeks post infection were plotted relative to the amount of virus used per well.

Antibodies

Primary antibodies used in these studies included rabbit anti-pChk2 (Thr68) (clone C13C1; Cell Signaling), rabbit anti-Chk2 (Cell Signaling), rabbit anti-pATR (Ser428) (Cell Signaling), rabbit anti-ATR (Cell Signaling), rabbit anti-pChk1 (Ser345) (clone 133D3; Cell Signaling), rabbit anti β-actin (Cell Signaling), mouse anti-ATM (clone 2C1; GeneTex, Irvine, CA), rabbit anti-KAP1 (GeneTex), rabbit anti-pKAP1 (S824) (Bethyl, Montgomery, TX), rabbit anti-pATM (Ser1981) (Clone EP1890Y, Abcam, Cambridge, UK), mouse anti-Chk1 (clone G-4; Santa Cruz, Heidelberg, Germany).

ACKNOWLEDGMENTS

We thank Micah Luftig for helpful discussion. Finally we wish to thank the patients and their parents for their cooperation.

CONFLICTS OF INTEREST

The authors disclose any potential conflicts of interest

GRANT SUPPORT

This work was supported by grants from the Swiss National Foundation (no. 310040–114118), the Cancer League of the Canton Zurich and the Wolfermann-Naegeli foundation.

REFERENCES

1. Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. Mol Cell. 2010; 40:179–204.
2. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science. 2003; 300:1542–1548.

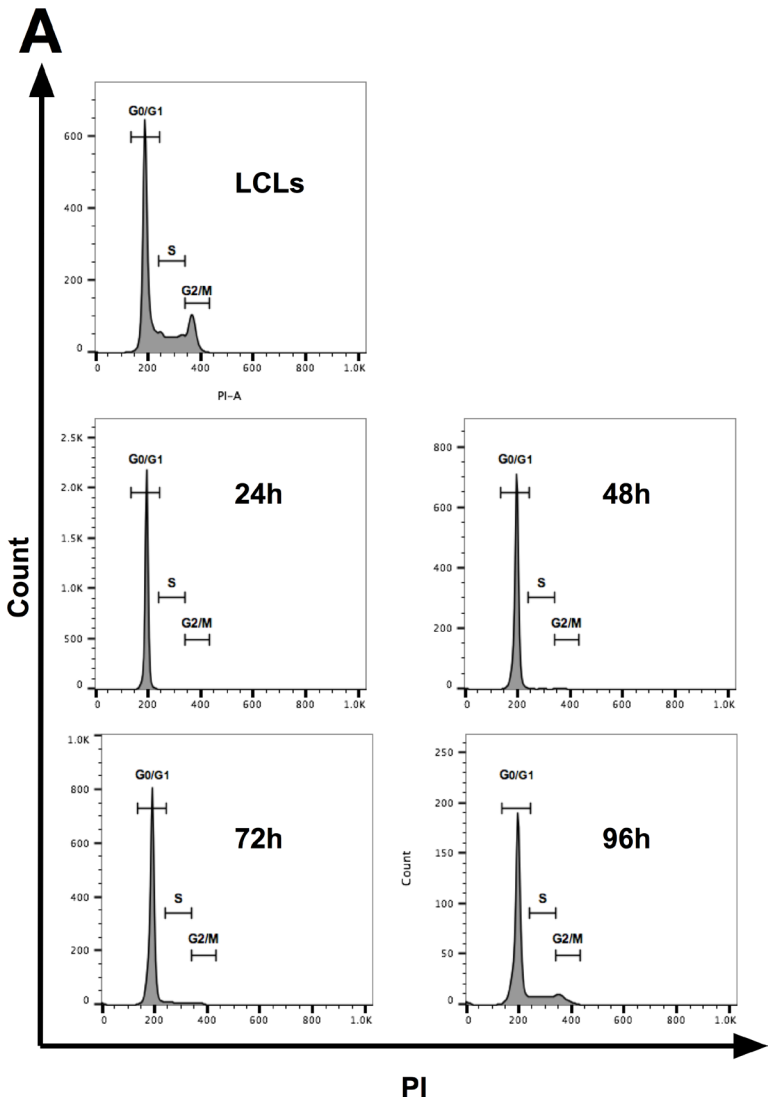
3. Dart DA, Adams KE, Akerman I, Lakin ND. Recruitment of the cell cycle checkpoint kinase ATR to chromatin during S-phase. J Biol Chem. 2004; 279:16433–16440.
4. Suzuki K, Kodama S, Watanabe M. Recruitment of ATM protein to double strand DNA irradiated with ionizing radiation. J Biol Chem. 1999; 274:25571–25575.
5. Lee JH, Paull TT. Activation and regulation of ATM kinase activity in response to DNA double-strand breaks. Oncogene. 2007; 26:7741–7748.
6. Smits VA, Reaper PM, Jackson SP. Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. Curr Biol. 2006; 16:150–159.
7. Wang'ondu R, Teal S, Park R, Heston L, Delecluse H, Miller G. DNA Damage Signaling Is Induced in the Absence of Epstein-Barr Virus (EBV) Lytic DNA Replication and in Response to Expression of ZEBRA. Plos One. 2015; 10.
8. Li R, Liao G, Nirujogi RS, Pinto SM, Shaw PG, Huang TC, Wan J, Qian J, Gowda H, Wu X, Lv DW, Zhang K, Manda SS, et al. Phosphoproteomic Profiling Reveals Epstein-Barr Virus Protein Kinase Integration of DNA Damage Response and Mitotic Signaling. PLoS Pathog. 2015; 11:e1005346.
9. Jha HC, Prasad AJM, Saha A, Banerjee S, Lu J, Robertson ES. Epstein-Barr Virus Essential Antigen EBNA3C Attenuates H2AX Expression. Journal of Virology. 2014; 88:3776–3788.
10. Luftig MA. Viruses and the DNA Damage Response: Activation and Antagonism. Ann Rev Virol. 2014; 1:605–625.
11. Nikitin PA, Yan CM, Forte E, Bocedi A, Tourigny JP, White RE, Allday MJ, Patel A, Dave SS, Kim W, Hu K, Guo J, Tainter D, et al. An ATM/Chk2-mediated DNA damage-responsive signaling pathway suppresses Epstein-Barr virus transformation of primary human B cells. Cell Host Microbe. 2010; 8:510–522.
12. Thorley-Lawson DA. Epstein-Barr virus: exploiting the immune system. Nat Rev Immunol. 2001; 1:75–82.
13. Rickinson A, Kieff E. . Epstein-Barr Virus. In: Knipe D and Howley P, eds. Fields Virology. (Philadelphia, PA: Lippincott Williams & Wilkins), pp. 2604–2654.
14. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. Nat Rev Cancer. 2004; 4:757–768.
15. Thorley-Lawson DA. EBV Persitence-Introducing the virus In: Münz C, ed. Epstein Barr Virus volume 1. (Switzerland Springer International Publishing), pp. 151–209.
16. Kuppers R. B cells under influence: transformation of B cells by Epstein-Barr virus. Nat Rev Immunol. 2003; 3:801–812.
17. Sugden B, Mark W. Clonal transformation of adult human leukocytes by Epstein-Barr virus. J Virol. 1977; 23:503–508.
18. Aman P, Ehlin-Henriksson B, Klein G. Epstein-Barr virus susceptibility of normal human B lymphocyte populations. J Exp Med. 1984; 159:208–220.
19. Rowe M, Lear AL, Croom-Carter D, Davies AH, Rickinson AB. Three pathways of Epstein-Barr virus gene activation from EBNA1-positive latency in B lymphocytes. J Virol. 1992; 66:122–131.
20. Crawford DH. Biology and disease associations of Epstein-Barr virus. Philos Trans R Soc Lond B Biol Sci. 2001; 356:461–473.
21. Thorley-Lawson DA, Gross A. Persistence of the Epstein-Barr virus and the origins of associated lymphomas. N Engl J Med. 2004; 350:1328–1337.
22. Taylor AL, Marcus R, Bradley JA. Post-transplant lymphoproliferative disorders (PTLD) after solid organ transplantation. Crit Rev Oncol Hematol. 2005; 56:155–167.
23. Dorner M, Zucol F, Berger C, Byland R, Melroe GT, Bernasconi M, Speck RF, Nadal D. Distinct *ex vivo* susceptibility of B-cell subsets to epstein-barr virus infection according to differentiation status and tissue origin. J Virol. 2008; 82:4400–4412.
24. Hawkins ED, Hommel M, Turner ML, Battye FL, Markham JF, Hodgkin PD. Measuring lymphocyte proliferation, survival and differentiation using CFSE time-series data. Nat Protoc. 2007; 2:2057–2067.
25. Gaillard H, Garcia-Muse T, Aguilera A. Replication stress and cancer. Nat Rev Cancer. 2015; 15:276–289.
26. Flynn RL, Zou L. ATR: a master conductor of cellular responses to DNA replication stress. Trends Biochem Sci. 2011; 36:133–140.
27. Lopez-Contreras AJ, Fernandez-Capetillo O. The ATR barrier to replication-born DNA damage. DNA Repair (Amst). 2010; 9:1249–1255.
28. Alonso-de Vega I, Martin Y, Smits VA. USP7 controls Chk1 protein stability by direct deubiquitination. Cell Cycle. 2014; 13:3921–3926.
29. Kaneko YS, Watanabe N, Morisaki H, Akita H, Fujimoto A, Tominaga K, Terasawa M, Tachibana A, Ikeda K, Nakanishi M. Cell-cycle-dependent and ATM-independent expression of human Chk1 kinase. Oncogene. 1999; 18:3673–3681.
30. Delia D, Fontanella E, Ferrario C, Chessa L, Mizutani S. DNA damage-induced cell-cycle phase regulation of p53 and p21waf1 in normal and ATM-defective cells. Oncogene. 2003; 22:7866–7869.
31. Smith J, Tho LM, Xu N, Gillespie DA. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. Adv Cancer Res. 2010; 108:73–112.
32. White D, Rafalska-Metcalf IU, Ivanov AV, Corsinotti A, Peng H, Lee SC, Trono D, Janicki SM, Rauscher FJ, 3rd. The ATM substrate KAP1 controls DNA repair in heterochromatin: regulation by HP1 proteins and serine 473/824 phosphorylation. Mol Cancer Res. 2012; 10:401–414.
33. Nikitin PA, Price AM, McFadden K, Yan CM, Luftig MA. Mitogen-induced B-cell proliferation activates Chk2-dependent G1/S cell cycle arrest. PLoS One. 2014; 9:e87299.
34. Pattengale PK, Smith RW, Gerber P. Selective transformation of B lymphocytes by E.B. virus. Lancet. 1973; 2:93–94.
35. Charrier JD, Durrant SJ, Golec JM, Kay DP, Knegtel RM, MacCormick S, Mortimore M, O'Donnell ME, Pinder JL,

Reaper PM, Rutherford AP, Wang PS, Young SC, et al. Discovery of potent and selective inhibitors of ataxia telangiectasia mutated and Rad3 related (ATR) protein kinase as potential anticancer agents. J Med Chem. 2011; 54:2320–2330.

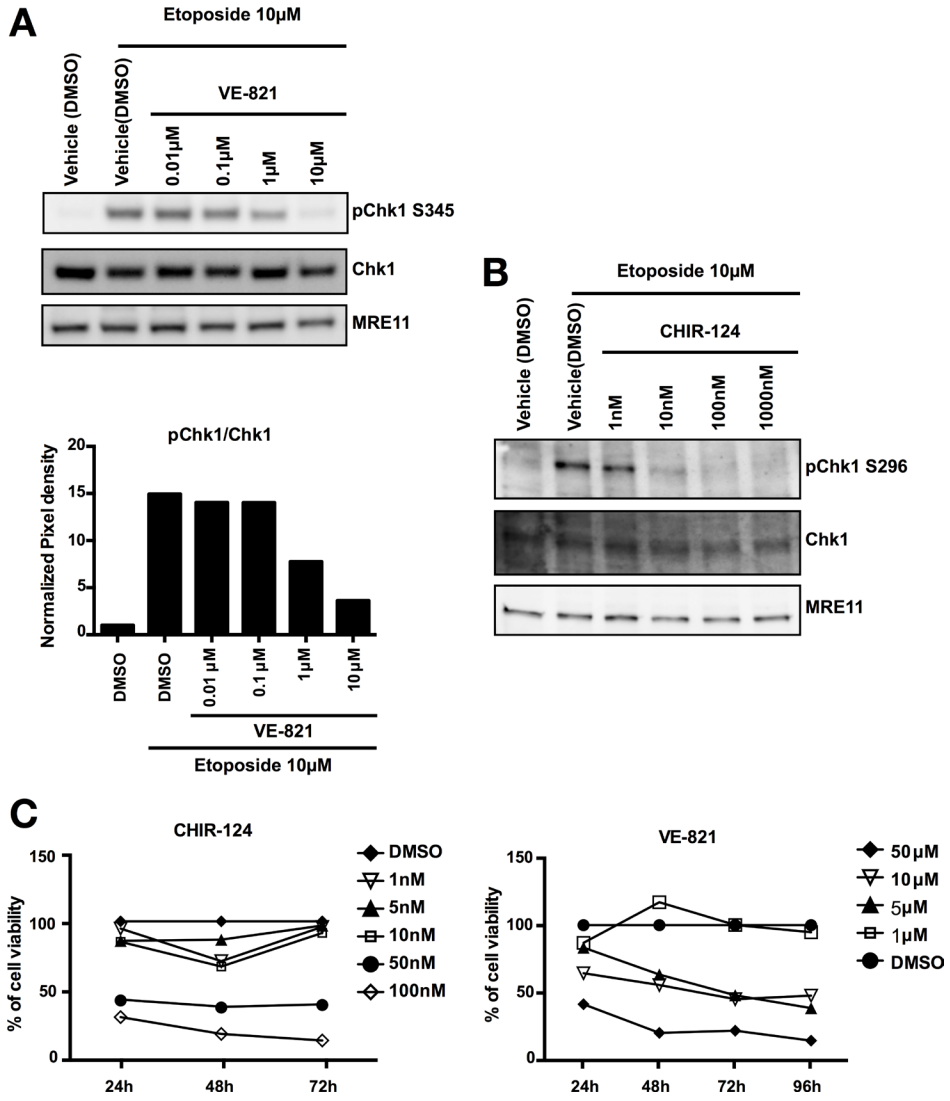
36. Tse AN, Rendahl KG, Sheikh T, Cheema H, Aardalen K, Embry M, Ma S, Moler EJ, Ni ZJ, Lopes de Menezes DE, Hibner B, Gesner TG, Schwartz GK. CHIR-124, a novel potent inhibitor of Chk1, potentiates the cytotoxicity of topoisomerase I poisons *in vitro* and *in vivo*. Clin Cancer Res. 2007; 13:591–602.
37. Kasahara K, Goto H, Enomoto M, Tomono Y, Kiyono T, Inagaki M. 14-3-3gamma mediates Cdc25A proteolysis to block premature mitotic entry after DNA damage. EMBO J. 2010; 29:2802–2812.
38. Li L, Gu B, Zhou F, Chi J, Wang F, Peng G, Xie F, Qing J, Feng D, Lu S, Yao K. Human herpesvirus 6 suppresses T cell proliferation through induction of cell cycle arrest in infected cells in the G2/M phase. J Virol. 2011; 85:6774–6783.
39. Goodnow CC, Vinuesa CG, Randall KL, Mackay F, Brink R. Control systems and decision making for antibody production. Nat Immunol. 2010; 11:681–688.
40. Zauner L, Melroe GT, Sigrist JA, Rechsteiner MP, Dorner M, Arnold M, Berger C, Bernasconi M, Schaefer BW, Speck RF, Nadal D. TLR9 triggering in Burkitt's lymphoma cell lines suppresses the EBV BZLF1 transcription via histone modification. Oncogene. 2010; 29:4588–4598.
41. Feederle R, Neuhierl B, Bannert H, Geletneky K, Shannon-Lowe C, Delecluse HJ. Epstein-Barr virus B95.8 produced in 293 cells shows marked tropism for differentiated primary epithelial cells and reveals interindividual variation in susceptibility to viral infection. Int J Cancer. 2007; 121:588–594.
42. Delecluse HJ, Hilsendegen T, Pich D, Zeidler R, Hammerschmidt W. Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. Proc Natl Acad Sci USA. 1998; 95:8245–8250.

Activation of ATR-Chk1 pathway facilitates EBV-mediated transformation of primary tonsillar B-cells

Supplementary Materials



Supplementary Figure S1: Cell cycle analysis of EBV inoculated TBCs. (**A**) Cell populations in G1, S, G2/M at different time points post EBV-inoculation were quantified by flow cytometry based cell cycle analysis measured by labeling the fixed cells with propidium iodide (PI). LCLs were used as positive control to determine the gate for G1-S-G2/M phases. Results shown are representative from 3 independent donors.



Supplementary Figure S2: Validation of the pharmacological inhibitors VE-821 or CHIR-124. (**A**) Inhibition of ATR was determined by measuring the expression of total Chk1 and pChk1 S345 by western blotting. Lymphoblastoid cell lines (LCLs) were treated 1 hour with Etoposide followed by a 2 hours treatment with the specific pharmacological ATR inhibitor VE-821. Chk1 phosphorylation at S345 was quantified by densitometric analysis. Data are represented as ratio of phosphorylated-to-total. Quantification was performed using the software imageJ 1.49 t. (**B**) Inhibition of Chk1 was determined by measuring the expression of total Chk1 and pChk1 S296 by western blotting. LCLs were treated 1 hour with Etoposide followed by a 2 hours treatment with the specific pharmacological Chk1 inhibitor CHIR-124. (**C**) Cell viability was measured by WST-1 assay as described in Materials and Methods. LCLs were exposed to different concentrations of VE-821 or CHIR-124 and cell viability was assessed at different time points.

IRAK4 is essential for TLR9-induced suppression of Epstein-Barr virus *BZLF1* transcription in Akata Burkitt's lymphoma cells

Marc Jordi[1, 2], Jeannine Marty[1, 2], Vanessa Mordasini[1, 2], Anna Lünemann[1, 2], Scott McComb[1, 2], Michele Bernasconi[1, 2], David Nadal[1, 2, 3]

[1]Laboratory for Experimental Infectious Diseases and Cancer Research of the Division of Infectious Diseases and Hospital Epidemiology and the Division of Oncology, University Children's Hospital of Zurich, University of Zurich, 8032 Zurich, Switzerland.

[2]Children's Research Center, University Children's Hospital of Zurich, University of Zurich, 8032 Zurich, Switzerland.

[3]Correspondence: Prof. Dr. D. Nadal, Division of Infectious Diseases and Hospital Epidemiology, University Children's Hospital, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland

E-mail: david.nadal@kispi.uzh.ch, Tel +41 44 266 72 50, Fax +41 44 266 80 72.

Abstract

Burkitt's lymphoma (BL) is the most common cancer in children of equatorial Africa, and is endemic to areas where people are chronically co-infected with Epstein-Barr virus (EBV) and the malaria pathogen *Plasmodium falciparum*. Although the contribution of both pathogens in the oncogenic process remains poorly understood, we showed earlier that the activation of Toll-like receptor (TLR) 9 by hemozoin, a disposal product formed from the digestion of blood by *P. falciparum*, suppresses the lytic reactivation of EBV in BL cells. The ability of latent EBV to evade the immune system, and its unique oncogenic potential, might thus be reinforced by co-infection with *P. falciparum*. Here, we explore in the BL cell line Akata the mechanism involved in repression of the viral transcription factor *BZLF1*, the master gene controlling EBV lytic reactivation, mediated by TLR9. We show that *BZLF1* repression is reinforced by TLR9 engagement via a mechanism that is largely independent of de novo protein synthesis. By CRISPR/Cas9-induced inactivation of TLR9, MyD88, IRAK4 and IRAK1 we confirm that this suppression is dependent on functional TLR9 and MyD88 signaling and identify IRAK4 to be essential for TLR9-induced suppression of *BZLF1* expression upon BCR cross-linking. Our results unprecedentedly show that TLR9-mediated inhibition of lytic EBV is largely independent of new protein synthesis and demonstrate the central roles of MyD88 and IRAK4 in this process contributing to EBV's persistence in the host's B-cell pool.

Importance

Latent Epstein-Barr virus (EBV) infection is associated with endemic Burkitt's lymphoma (eBL) that is epidemiologically linked to chronic infection with the malaria agent *Plasmodium falciparum*. Lytic reactivation of EBV eventually leads to the death of the host cell. Previously, we showed that activation of the innate immunity signaling via Toll-like receptor (TLR) 9 by hemozoin, a pigment of *P. falciparum,* is critical in inhibiting EBV lytic reactivation and thereby causing reinforcement of its latency in eBL cells. Here, we show that EBV lytic repression is reinforced by TLR9 engagement via a mechanism that is largely protein synthesis independent and that two TLR9 downstream signaling molecules, MyD88 and IRAK4, exhibit central roles. Counteracting signaling of these molecules could allow lytic reactivation and thus provide novel treatment targets to combat eBL.

Introduction

Primary infection with the Epstein-Barr virus (EBV) is mostly asymptomatic, and more than 90% of the adult population worldwide are EBV carriers after the virus has established reversible latent infection (1, 2). This life-long, virtually harmless, host–virus coexistence must be regarded as the result of a long co-evolution based on modulation of EBV gene expression in different subsets of infected cells and the finely tuned adaptation to the immune response of the human host (3). Yet, EBV is associated with endemic Burkitt's lymphoma (eBL), one of the most common cancers in children in equatorial Africa, i.e., in areas where chronic co-infection with EBV and the malaria parasite *Plasmodium falciparum* prevails (4). As a member of the gammaherpesvirus family, EBV establishes latency in B cells (5). In eBL cells, EBV persists in a highly restricted form of latency (6), termed latency program I. In this program, EBV's lytic and latent genes are repressed except EBV nuclear antigen (EBNA)1, which is essential for episomal retention of EBV in dividing cells. Thereby, the propagation of the virus to daughter cells is guaranteed and the repression of EBV's gene expression contributes to the evasion from the host's immune system (7).

Latency of EBV is reversible, to ensure viral transmission to uninfected cells and to new hosts (2). Thus, EBV periodically lytically reactivates, which leads to the production of infectious viral particles and death of the infected B-cell. The lytic reactivation is set off by the expression of the immediate-early protein ZEBRA encoded by EBV's master lytic gene *BZLF1*. ZEBRA is a transcription factor that induces a lytic cascade leading to early and late lytic EBV gene expression (8). In BL cell lines, latently infected with EBV (e.g. Akata cells), the lytic reactivation can be induced using diverse agents including phorbol esters (TPA), sodium butyrate (SB), transforming growth

101 factor-β (TGF-β), and B-cell receptor (BCR) cross-linking anti-immunoglobulin G (anti-
102 IgG) (9–13). EBV particles and lytic proteins provoke a wide range of immune responses
103 through innate immune mechanisms (14) and adaptive humoral (15) and T-cell
104 responses (16). Thus, the restriction to essential proteins required for the latent viral
105 persistence and replication is beneficial for both EBV and the host cell. To prevent
106 aberrant spontaneous lytic reactivation, cell death and subsequent activation of the
107 immune system, EBV latency is tightly controlled by histone modifications (17, 18) and
108 by DNA methylation (19). In addition to these intrinsic regulating factors, EBV can hijack
109 the innate immune system, and, in particular, the signaling via Toll-like receptors (TLRs)
110 to regulate the balance between latency and lytic reactivation (20). Our group showed
111 that hemozoin, a disposal product formed from the digestion of blood by *P. falciparum*,
112 activates TLR9 signaling and suppresses the lytic reactivation of EBV in BL B-cells *in*
113 *vitro* by affecting the histones state at the promoter of *BZLF1* (21). Nevertheless, it
114 remains unclear whether this effect is protein synthesis independent, thus a direct
115 consequence of the activation of TLR9 signaling, or whether it requires the expression of
116 a specific unknown protein.
117 TLRs are essential elements of the innate immune system. They are
118 transmembrane receptors involved in the recognition of pathogen associated molecular
119 patterns (PAMPs) or danger associated molecular patterns (DAMPs), which initiate the
120 inflammatory response by the production of cytokines (22, 23). Endosomal TLR9 is
121 expressed in B cells and acts as a sensor for unmethylated CpG oligonucleotides (ODN)
122 found on a large scale in bacterial DNA (24). Upon stimulation, the TLR9 cytoplasmic
123 Toll/interleukin-1 receptor (TIR) domain associates with the TIR domain-containing
124 adaptor myeloid differentiation primary response gene 88 (MyD88). The latter recruits
125 the interleukin-1 receptor-associated kinase (IRAK) 4 to TLR9 through interaction of the

death domains of both molecules. IRAK-1 is activated by phosphorylation and associates with the TNF receptor associated factor (TRAF) 6, thereby activating the IκB kinase (IKK) complex, leading to activation of mitogen-activated protein (MAP) kinases (JNK, p38, MAPK) and of nuclear factor kappa B (NF-κB). NF-κB promotes the transcription of genes involved in the cellular activation, proliferation and in the production of pro-inflammatory cytokines (25). Recently, we showed that several elements of the TLR9 signaling pathway, including NF-κB, PI3K, ERK, JNK and p38, are not necessary for the inhibitory effect of TLR9 signaling on *BZLF1* mRNA expression (21). Thus, additional investigation is required to precisely delineate how TLR9 signaling impacts on EBV lytic reactivation. Understanding the mechanisms favoring maintenance of lytic EBV infection could provide potential targets for treatments aiming at activating lytic EBV replication and inducing lysis of EBV-harboring cancer B cells.

Here, we aimed at advancing the detailed understanding of TLR9 stimulation suppressing EBV lytic reactivation. Using protein synthesis inhibitors, we investigated whether inhibition of EBV lytic reactivation requires *de novo* protein expression, or if it runs through already existing elements. Moreover, we tested the importance of key components of the signaling pathway, which are directly downstream of TLR9 by generating Akata BL cells with either silenced or inactivated *TLR9*, *MyD88*, *IRAK4,* or *IRAK1* genes.

Material and Methods

Cells and cell culture

The BL cell line Akata (12) was obtained from Dr. Andrew Bell (Birmingham, UK). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, streptomycin (100 mg/ml), penicillin (100 U/ml) and L-glutamine (2 mM). Akata cells transfected with the shLucGL3 (control) or shIRAK4 expressing plasmids were grown in complete medium supplemented with 200 µg/ml Zeocin (InvivoGen, Nunningen, Switzerland). Akata cells expressing a dominant-negative MyD88 (DN-MyD88 Akata) (21) were grown in the same medium supplemented with 0.4 mg/ml G418 (Promega, Dübendorf, Switzerland).

Reagents and antibodies

ODN 2006 type B CpG oligonucleotide (# 11B15-MM) was bought from InvivoGen. The polyclonal rabbit α-human-IgG antibody (#A0423) was obtained from Dako (DakoCytomation, Zug, Switzerland). Cycloheximide (VWR international, Dietikon, Switzerland) was dissolved in H_2O to a concentration of 10 mg/ml, whereas 4E1RCat (Sigma-Aldrich, Buchs, Switzerland) was adjusted to a concentration of 500 µg/ml in DMSO. Digitonin was dissolved in DMSO to a concentration of 20 mg/ml.

EBV lytic reactivation and ODN CpG 2006 stimulation

Akata cells were resuspended at 1 x 10^6 cells/ml in supplemented RPMI 1640 and stimulated with 0.5 mM end concentration ODN CpG 2006 (InvivoGen) 2 h prior to stimulation with 100 µg/ml anti-IgG (Dako). At 6 h after treatment with anti-IgG, cell pellets were harvested for RNA and protein extraction.

172

173 *Protein synthesis inhibition*

174 For protein synthesis inhibition, Akata cells were treated with 4E1RCat (10 μM or 25
175 μM) or cycloheximide (33 μg/ml) for 30 min before ODN CpG 2006 treatment. Cells
176 were then treated as described in the section above. The cells viability was assed by
177 Trypan Blue exclusion assay.

178

179 *hIL-10 ELISA*

180 hIL-10 protein concentrations were determined in supernatants from stimulated
181 cultures by standard capture ELISA (Ready- SET-Go, eBioscience, Vienna, Austria)
182 according to the manufacturer's instructions. Plates were read using a Synergy HT Multi-
183 Detection Microplate Reader (BioTek, Luzern, Switzerland) at 450 nm and 570 nm. The
184 values measured at 570 nm were subtracted from those of 450 nm and the cytokine
185 concentration was determined by extrapolation from the standard curve.

186

187 *RNA preparation, reverse transcription and RT-qPCR (TaqMan)*

188 RNA isolation, DNAse treatment, reverse transcription and quantitative PCR (RT-qPCR)
189 was performed as described before (20, 26). *BZLF1*, *hIL-10*, *C-myc* (Hs00153408_m1;
190 Life Technologies, Zug, Switzerland), *IRAK4* (Hs00211616_m1; Life Technologies) or
191 *TLR9* (Hs00152973_m1; Life Technologies) mRNA expression was normalized to the
192 mRNA of the housekeeping gene *HMBS* (same as above) resulting in Δ cycle threshold (
193 ΔCT) values.

194

195 *Cell lysis and Western blot*

Total protein lysates were obtained after lysing 10^6 cells in RIPA complete buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 2mM EDTA, 1% NP40 complemented with 0.1% SDS, 1 x EDTA-free protease inhibitor cocktail (Roche, Rotkreuz, Switzerland)). Cell extracts were passed 10 times through a 25-G syringe. Protein content was determined using the Pierce BCA Protein Assay Kit (ThermoScientific, Zug, Switzerland), according to the manufacturer's instructions. To analyze protein expression by western blot, protein (20 mg/well) was loaded into a NuPAGE 4–12% Bis-Tris Gel (Life Technologies), subjected to SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare, Glattbrugg, Switzerland). The membrane was incubated with rabbit anti-IRAK1 (sc-7883, Santa Cruz Biotech, Santa Cruz, CA), rabbit anti-Akt (#9272), rabbit anti-pAkt (#4058), rabbit anti-Mek1/2 (#9122), rabbit anti-pMek1/2 (#2338), rabbit anti-Syk (#12358), rabbit anti-pSyk (#2710) or rabbit anti-β-actin (# 4967, all from Cell Signaling Technology, Allschwil, Switzerland) primary antibodies; subsequently with anti-rabbit (# 7074) or anti-mouse (# 7076) IgG HRP-linked secondary antibodies (Cell Signaling Technology). The signal was detected with the ECL Western Blotting Detection Reagents (GE Healthcare) and imaged using the LAS-3000 image reader (Fujifilm, Dielsdorf, Switzerland).

EBV genome measurement

Genomic DNA was extracted from 2 x 10^6 Akata cells using the QIAamp DNA Mini Kit (Qiagen, Hombrechtikon, Switzerland). Primers were diluted to a concentration of 300 nM with 150 ng DNA, 1x SYBR green MasterMix (#4309155, Life Technologies) and filled with water to a volume of 10 µl. Samples were measured in triplicates. Measurements were carried out on an ABI 7900HT Fast real-time PCR system (Applied

Biosystems, Rotkreuz, Switzerland) and analyzed with SDS 2.2 software. SYBR green primers were as following:

genomic BamH1 W: forward: GCCAGACAGCAGCCAATTGT; reverse: GACTCCTGGCGCTCTGATG*; genomic HMBS*: forward: ACCAGCTCCCTGCGAAGAG; reverse: GAACTCCAGATGCGGGAACTT

CRISPR/Cas9 genome editing

Single guide RNA sequences targeting IRAK4, IRAK1, MyD88 and TLR9 were designed using the CRIPR Design webtool (http://crispr.mit.edu/). The three sequences having the lowest number of off-target sites were selected for each gene (**Table 1, Figure 1**). Complementary oligonucleotides containing the guide RNA (without PAM sequence) and BpiI ligation adapter were synthesized by Microsynth (Balgach, Switzerland). The annealed oligos were ligated into BpiI digested pSpCas9(BB)-2A-GFP (px458) vector, a gift from Feng Zhang (Addgene plasmid # 48138). The sequence of the constructs was verified by DNA sequencing. CRISPR single cell clones were obtained by electroporation (Neon® Transfection system, ThermoFisher Scientific, Zug, Switzerland) of Akata cells with the px458 plasmids. Transfected single cells, positive for green fluorescent protein (GFP), were sorted 48h after transfection using a FACS ARIA II cell sorter (BD Biosciences, Allschwil, Switzerland). Single cell clones were genotyped and characterized after expansion (**Table 2, Figure 1**).

Statistics

Level of significance was evaluated by an unpaired Student's *t* test or Tukey's multiple comparison test (ANOVA) using Prism 6 (GraphPad Software, Inc.). P levels < 0.05 were regarded as statistically significant.

245

246

Results

TLR9 pathway activation by ODN CpG 2006 does not require protein synthesis to inhibit BZLF1 expression

Hemozoin, a pigment expressed by the malaria agent *Plasmodium falciparum*, activates the TLR9 pathway (21, 27). We have previously shown that TLR9-triggering by ODN CpG 2006 or Hemozoin inhibits EBV lytic reactivation, i.e. expression of *BZLF1*, induced by BCR cross-linking in BL cells *in vitro* (21). Here, we aimed to elucidate the mechanism by which TLR9 inhibits *BZLF1* expression. In particular, we wanted to understand if *BZLF1* expression is inhibited directly e.g. through post-translational modification of existing proteins induced by the TLR9 signaling, or indirectly, through *de novo* expression of proteins, e.g. of a transcription or repressor factor expressed upon TLR9 stimulation.

We treated Akata BL cells with a protein synthesis inhibitor, either cycloheximide or 4E1RCat. We chose these inhibitors because they act at the translational level and should not influence *BZLF1* mRNA levels. First, in order to determine the cytotoxicity of the protein synthesis inhibitors, we assessed the cell viability 8.5h after treatment of Akata BL cells with 33 μg/ml of cycloheximide, or 10 μM and 25 μM of 4E1RCat, respectively (**Fig. 2a**). Treatment with 33 μg/ml cycloheximide, or 10 μM 4E1RCat did not affect cell viability, whereas 25 μM 4E1RCat or vehicle treatment, with the same volume DMSO as for 25 μM 4E1RCat, decreased cell viability compared to untreated cells to 60% ($p=0.554$), and to 73% ($p=0.23$), respectively. The treatment of the cells with digitonin, used as positive control, significantly reduced the cell viability to 30% ($p=0.0034$) (**Fig. 2a**)..

Akata BL cells first treated with protein synthesis inhibitors, cycloheximide or 4E1RCat for 30 min, were tested for EBV lytic reactivation after additional treatment with ODN CpG 2006 or/and anti-IgG. After 6h treatment with anti-IgG, EBV lytic reactivation was assessed by the measurement of the increase of the mRNA expression of the immediate early lytic transcription factor *BZLF1* (**Fig. 2b and c**). Treatment with ODN CpG 2006, cycloheximide or 4E1RCat had *per se* no effect on *BZLF1* mRNA expression levels (**Fig. 2b**). In cells with no protein synthesis inhibition, activation of TLR9 with ODN CpG 2006 inhibited the *BZLF1* mRNA increase after BCR cross-linking with anti-IgG, and thus lytic reactivation, by 83.7% (p=0.0317) (**Fig. 2c**). Treatment with cycloheximide or 4E1RCat led to respectively a 14-times and to an 11-times lower *BZLF1* mRNA expression after BCR triggering via anti-IgG compared to cells with no protein synthesis inhibition. This was expected, since expression of *BZLF1* upon BCR crosslinking is reinforced by a positive feedback loop exerted by ZEBRA, the protein expressed by the *BZLF1* gene, on its own promoter (29–31) (**Fig. 2c**). Importantly, upon protein synthesis inhibition with cycloheximide or 10 μM 4E1RCat, TLR9 triggering via ODN CpG 2006 inhibited *BZLF1* mRNA increase by 77.7% (p=0.0418) and by 78.7 % (p=0.011), respectively. Therefore, TLR9 stimulation can inhibit *BZLF1* expression directly through a largely protein synthesis independent mechanism.

TLR9-induced inhibition of EBV lytic reactivation is MyD88, IRAK4 and IRAK1 dependent in Burkitt's lymphoma cells

As shown above, TLR9 activation triggered inhibition of EBV lytic reactivation does not require *de novo* protein synthesis and is strictly MyD88-dependent (21). However, the downstream TLR9 signaling elements, NF-κB, phosphatidylinositol-3 kinase (PI3K),

297 extra-cellular signal regulated kinase (ERK), *c-jun* N terminal kinase (JNK) and p38, are
298 not fully responsible for TLR9-dependent *BZLF1* inhibition (21). IRAK4 and IRAK1
299 molecules, which act immediately downstream of MyD88, were not tested. Thus, to
300 know the importance of IRAK4 and IRAK1 proteins in TLR9-induced inhibition of *BZLF1*
301 expression, we used the CRISPR/Cas9 genome editing method (32) to inactivate IRAK4
302 and IRAK1, respectively. In separate experiments, TLR9 and MyD88 were also
303 inactivated to further confirm our previous results, and to use the TLR9 or MyD88
304 inactivated cells as positive controls.

305 After transfection of Akata cells with modified Cas9-gRNA-GFP (px458)
306 expression plasmids (**Fig. 1**), GFP-positive single cells were sorted and amplified. The
307 clones were sequenced and 2-3 clones containing an early stop codon in the TLR9,
308 MyD88, IRAK4, or IRAK1 coding sequences were selected for further characterization
309 (**Table 2**). To verify that every cell clone had the same receptors expression level, IgG
310 protein expression on the cells' surface was characterized by flow cytometry (**Fig. 3a**),
311 and for TLR9 mRNA levels by were measured by RT-qPCR (**Fig. 3b**). We then measured
312 IL-10 protein levels in the supernatants (**Fig. 3c**) and the NF-κB nuclear translocation
313 (**Fig. 4**), after 8h treatment with ODN CpG 2006, as readouts for the integrity of the TLR9
314 pathway activity. Finally, we amplified the conserved EBV *BamHI W* sequence, present
315 in multiple copies on the viral genome, by RT-qPCR to determine the EBV presence in
316 the cell clones.

317 None of the inactivating mutations did affect the BCR surface expression levels of
318 the cloned cells as determined by flow cytometry. 81.1% of the Akata WT cells
319 expressed IgG on the surface, whereas 90.4 % of the IRAK4$^-$ a3, 94.3 % of the IRAK4$^-$ a4,
320 92.3 % of the IRAK1$^-$ b2, 93.1 % of the IRAK1$^-$ b8, 91.8 % of the TLR9$^-$ b3, 91.7 % of the
321 TLR9$^-$ b5, 92.7 % of the TLR9$^-$ b6, 92.9 % of the MyD88$^-$ a3, 94.2 % of the MyD88$^-$ a5 and

95.3 % of the MyD88$^-$ b1 cell clones were IgG positive (**Fig. 3a**). The *TLR9* mRNA levels in the clones ranged between 0.5-fold and 1.2-fold of the mRNA levels measured in the Akata WT cells (**Fig. 3b**). On the other hand, the 5.5-fold IL-10 protein level increase measured after 8h ODN CpG 2006 treatment of WT Akata cells was not observed in the IRAK4$^-$ a3, IRAK4$^-$ a4, IRAK1$^-$ b2, IRAK1$^-$ b8, TLR9$^-$ b3, TLR9$^-$ b5, TLR9$^-$ b6, MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 cell clones, (**Fig. 3c**) indicating that the inactivation of the TLR9 pathway was not a consequence of the variation in TLR9 mRNA levels. We confirmed the inactivation of the TLR9 pathway by measuring the NF-κB nuclear translocation by WB. After 90 min treatment with ODN CpG 2006, Akata WT cells showed a strong nuclear NF-κB protein levels increase compared to untreated cells, on the other hand in IRAK4$^-$ a3, IRAK4$^-$ a4, TLR9$^-$ b3, TLR9$^-$ b5, TLR9$^-$ b6, MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 cell clones the nuclear NF-κB was not increased by ODN CpG 2006 treatment (**Fig. 4**). The selected cell clones IRAK4$^-$a3, IRAK4$^-$a4, TLR9$^-$b3, TLR9$^-$b5, TLR9$^-$b6, MyD88$^-$a3, MyD88$^-$a5 and MyD88$^-$b1 contained between 20 and 95 *BamHI W* copies per cell. For comparison the Akata WT cells contained about 85 *BamHI W* copies (**Fig. 3d**).

Despite variable EBV DNA copy numbers per cell, the detection of *BamHI W* copies allowed us to use the cell clones IRAK4$^-$ a3, IRAK4$^-$ a4, TLR9$^-$ b3, TLR9$^-$ b5, TLR9$^-$ b6, MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 for further *BZLF1* expression assays. By contrast and surprisingly, the *BamHI W* sequence was not detectable in IRAK1 cell clones IRAK1$^-$ b2 and IRAK1$^-$ b8, kept about 1 month in culture for the amplification step after single cell cloning. For this reason, the *BZLF1* reactivation upon BCR cross-linking was not detectable anymore in the IRAK1$^-$ clones. The transfection, selection and amplification steps were therefore repeated using IRAK1$^-$b px458 plasmid. In order to control that the loss of EBV copies in IRAK1 inactive clones was not due to the long

culture time required for the amplification step, Akata cells transfected with a control px458 plasmid lacking a sgRNA target sequence, as well as untransfected Akata WT cells were isolated and amplified in parallel. The cells were directly tested for the TLR9 pathway activity by the measurement of IL-10 mRNA and proteins in the supernatant, as well as for the presence of EBV by the measurement of *BamHI W* copies (**Table 3**). To sum up the CRISPR/Cas9 plasmid transfection experiments, Akata cells transfected with control px458 plasmid as well as untransfected Akata single cell clones all had a active TLR9 pathway and about 83.3% of the clones where EBV positive. This shows that the control px458 plasmid and the transfection, selection and amplification steps necessary to obtain single cell clones had no influence on the TLR9 pathway activity and the EBV presence in the cells. On the other hand, respectively 56.5%, 40%, 50% and 60% of the IRAK1^{-}, IRAK4^{-}, TLR9^{-} and MyD88^{-} px458 transfected clones had an inactive TLR9 pathway. Interestingly, respectively 75%, 60% and 83.3% of the IRAK4^{-}, TLR9^{-} and MyD88^{-} px458 transfected clones that had an inactive TLR9 pathway and were EBV positive (**Table 3**). In IRAK1^{-}b px458 transfected cell clones, about 56.5 % of the clones had an inactive TLR9 pathway, and all had completely lost EBV genome (**Table 3**), which could suggests a role for IRAK1 in EBV viral persistence and transmission to dividing daughter cells. For this reason, the *BZLF1* reactivation upon BCR cross-linking was not detectable anymore in the IRAK1^{-} clones.

Next, in order to determine the importance of IRAK4 in the TLR9-induced inhibition of *BZLF1* expression the CRISPR/Cas9 mutated clones were treated with ODN CpG 2006 for 2h, followed by treatment with anti-IgG for 6h. TLR9 and MyD88 knockouts were used as controls. EBV lytic reactivation was determined by the measurement of *BZLF1* mRNA levels by RT-qPCR. For the IRAK4 inactive clones IRAK4^{-} a3 and IRAK4^{-} a4, we measured 194 and 463 *BZLF1* relative transcripts over *HMBS* over

untreated sample after BCR cross-linking, respectively (**Fig. 5a**); and for the TLR9 inactive clones TLR9$^-$ b3, TLR9$^-$ b5 and TLR9$^-$ b6, we measured 789, 627 and 940 *BZLF1* relative transcripts over *HMBS* over untreated sample after BCR cross-linking, respectively (**Fig. 5b**). For comparison, for WT Akata cells we measured 1583 *BZLF1* relative transcripts over *HMBS* over untreated sample after BCR cross-linking. In the MyD88 inactive clones MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 we measured 1623.8, 1632 and 1837 *BZLF1* relative transcripts over *HMBS* over untreated sample after BCR cross-linking, respectively (**Fig. 5c**).

The basal *BZLF1* mRNA levels measurement in untreated and ODN CpG 2006 treated cells shows that the differences of spontaneous lytic reactivation between the TLR9-, MyD88- and IRAK4- CRISPR/Cas9 clones compared to WT Akata cells, are minor, although statistically significant for TLR9-b5, MyD88-a3 and MyD88-b1 clones (**Supplementary Fig. S1**). Moreover, the differences in basal *BZLF1* mRNA levels do not correlate with the EBV DNA copies measured in each clones (**Fig. 3d**) and neither with the EBV lytic reactivation upon BCR cross-linking (**Fig. 5**). Spontaneous lytic reactivation is therefore, not the reason explaining neither the differences in EBV DNA loads nor the differences in *BZLF1* mRNA expression upon BCR cross-linking.

In order to investigate if the BCR signaling is affected by the mutation we stimulated the IRAK4$^-$ a3, IRAK4$^-$ a4, TLR9$^-$ b3, TLR9$^-$ b5 and TLR9$^-$ b6 clones with anti-IgG for 15, 30, 60 and 120 minutes. The phosphorylation levels of Akt, Mek and Syk were measured in stimulated CRISPR/Cas9 clones by western blot and compared with the levels in Akata WT cells. The results show an increased Akt, Mek and Syk phosphorylation upon treatment with anti-IgG with only minor differences between the IRAK4$^-$ and TLR9$^-$ clones and the Akata WT cells (**Supplementary Fig. S2, S3 and S4**). From these results we exclude an influence of IRAK4 or TLR9 abrogation on the BCR

pathway to be the reason for the differences in *BZLF1* mRNA expression upon lytic reactivation.

The activation of TLR9 by ODN CpG 2006 in WT Akata cells reduced the expression of *BZLF1* mRNA about 4.5 times to 379.7 (p=0.0005) relative transcripts after BCR cross-linking. Interestingly, we showed that, despite differences in the *BZLF1* mRNA level after BCR cross-linking between the clones and WT Akata cells, the TLR9-induced reduction of *BZLF1* mRNA expression was completely rescued in the IRAK4$^-$a3 and IRAK4$^-$a4 clones with 264.6 (p=0.0363) respectively 527.3 (p=0.1855) *BZLF1* relative transcripts (**Fig. 5a**); and in the TLR9$^-$b3, TLR9$^-$b5 and TLR9$^-$b6 control clones, with respectively 855.5 (p=0.0112), 527.2 (p=0.078) and 998.1 (p=0.2435) *BZLF1* relative transcripts (**Fig. 5b**); and in the MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 control clones with respectively 1408.6 (p=0.2998), 1861.2 (p=0.3735) and 1682.9 (p=0.064) *BZLF1* relative transcripts (**Fig. 5c**). To sum up, by transfecting Akata cells with CRISPR/Cas9 plasmids containing sgRNA targeting IRAK4, TLR9 or MyD88 genes, insertions or deletions could be induced leading to an early stop codon in the target genes. The characterization of the single cell clones showed that the surface IgG protein level remained stable and comparable to the levels measured in WT Akata cells; that the TLR9 pathway activity was completely abrogated by the early stop codon in the target genes; and not as a consequence of the variation in TLR9 mRNA level; finally, despite variable EBV DNA copy numbers per cell, the IRAK4$^-$ a3, IRAK4$^-$ a4, TLR9$^-$ b3, TLR9$^-$ b5, TLR9$^-$ b6, MyD88$^-$ a3, MyD88$^-$ a5 and MyD88$^-$ b1 clones could be used for lytic reactivation assays.

In conclusion, these results show that in BL cells the TLR9-induced inhibition of lytic reactivation requires functional IRAK4, TLR9 and MyD88 proteins.

Discussion

EBV lytic reactivation upon BCR cross-linking is inhibited by TLR9 stimulation with ODN CpG 2006 in B cells (21). Here, we studied the importance of protein synthesis, and the involvement of the signaling proteins MyD88, IRAK4 and IRAK1, in this process. We found that (i) TLR9-induced inhibition of EBV lytic reactivation in B-cells upon BCR cross-linking, is largely independent from de novo protein synthesis; (ii) the inactivation of either TLR9, MyD88, or IRAK4 completely abrogates the effect of ODN CpG 2006 on TLR9 signaling resulting in unhindered EBV lytic reactivation upon BCR cross-linking in the presence of ODN CpG 2006. Our results unprecedentedly show that TLR9 triggering activates a signaling pathway that does not only depend on protein synthesis to favor EBV latency in B cells upon stimulation of the BCR; and demonstrate the central roles of MyD88 and IRAK4 in this mechanism contributing to EBV's persistence in the host's B-cell pool.

Remarkably, activation of TLR9 by ODN CpG 2006 inhibited the transcription of EBV's master lytic gene *BZLF1* in BL cells to a similar degree, even when mRNA translation was strongly inhibited. To direct our investigation, we tackled the question whether protein synthesis is decisive in EBV's reactivation process upon challenges imposed to the host cells. In Akata BL cells the strong increase of ZEBRA expression upon BCR cross-linking, the protein coded by *BZLF1*, goes through a bi-phasic process that requires protein synthesis to be amplified. (29). Protein synthesis inhibitors are therefore expected to abrogate the positive feedback loop. In fact, upon BCR cross-linking, we observed lower *BZLF1* mRNA expression levels in cells treated with cycloheximide or 4E1RCat. Interestingly, TLR9 stimulation with ODN CpG 2006 inhibited *BZLF1* mRNA expression at the same ratio, whether protein synthesis was

447 inhibited or not. This strongly suggests that the regulation of EBV's lytic reactivation
448 upon TLR9 stimulation with ODN CpG 2006 is, to a large extend, protein synthesis
449 independent and suggests post-translational modifications to be responsible for EBV
450 lytic gene repression. The question weather TLR9 activation also affects ZEBRA's
451 activity and the positive amplification ZEBRA exerts on its own promoter remains open.
452 Our group revealed that TLR9 triggering affects histone modifications, which is, at least
453 partly, responsible for EBV lytic gene repression (21). In addition, several studies
454 showed that epigenetic mechanisms including histone modifications or DNA
455 methylation are responsible for the regulation of EBV lytic gene expression upon BCR
456 cross-linking (36, 37). Consequently, even if we cannot completely exclude the existence
457 of a newly synthesized unknown repressor factor(s), our results strongly indicate that
458 EBV reinforces its latency by hijacking the TLR9-induced post-translational
459 modifications to repress its lytic genes expression.
460 We unequivocally and unprecedentedly show that MyD88 and IRAK4 are
461 essential for the TLR9-dependent inhibition of EBV lytic reactivation upon BCR cross-
462 linking. This was possible after we inactivated *TLR9*, *MyD88*, or *IRAK4* genes in single
463 cell clones by CRISPR/Cas9 methodology. Importantly, we proved that the canonical
464 TLR9 signaling pathway was completely abrogated by the inactivating mutations as
465 revealed by the lower IL-10 concentrations in the supernatant and the missing NF-κB
466 nuclear translocation after stimulation with ODN CpG 2006. Of note, we excluded that
467 *TLR9, MyD88,* or *IRAK4* inactivation affects the degree of BCR expression on the cell
468 surface. Thus, the TLR9-induced abrogation of the effects subsequent to BCR-cross-
469 linking is not due to changes in BCR expression degree.
470 Our findings contribute to an increased understanding of the mechanisms
471 involved in the TLR9-induced inhibition of EBV lytic reactivation and confirm our

previously reported observations (21). Moreover, as reported earlier, TLR9-mediated suppression of EBV lytic gene expression is not restricted to latently infected BL cells but can also be observed *ex vivo* in primary cells upon EBV infection (20). It seems likely that this feature is shared by other gammaherpesviruses, as we found that stimulation of TLR9, as well as of TLR7, similarly suppresses spontaneous and induced MHV-68 reactivation in mice (41). In addition, NF-κB nuclear translocation was shown to suppress the lytic genes of the Kaposi's sarcoma-associated herpesvirus (42). Thus, gammaherpesviruses seem to have evolved to exploit cellular signaling mechanisms in order to keep their latent state and avoid unnecessary aberrant lytic reactivation. The infectious viral particles produced upon EBV lytic reactivation are recognized by the host's immune system and lead to an inflammatory response that could be harmful for both the virus and the host (43). Our findings indicate that EBV developed a very strong and efficient strategy, by taking advantage of the host cells innate immune TLR9 signaling machinery through MyD88 and IRAK4, to ensure and maintain its reversible latency.

Most intriguingly, we failed to establish *IRAK1* mutated BL cell clones retaining EBV genomes. Since the 3 CRISPR plasmids we developed were efficient in mutating *IRAK1* gene in at least one single cell clone, this preliminary finding suggests that IRAK1 could play a central role in steering EBV persistence. EBV nuclear protein EBNA1 binds to the origin of viral replication and is essential to mediate replication and partitioning of its episome during the division of the host cell. EBNA1 function is regulated by the phosphorylation of ten specific sites (44). The kinases responsible for EBNA1 phosphorylation have not been identified, but IRAK1 could be a potential candidate. Thus, one hypothesis for the loss of EBV in the *IRAK1*-mutated clones is that EBNA1's function is altered by the inactivation of IRAK1, which affects the replication and

partitioning of EBV's genomic DNA in dividing cells leading to the dilution of the virus in the daughter cells and finally to its loss. However, it is important to consider that these result has only been observed in a limited number of clones, mostly transfected with the IRAK1^{-}b CRISPR/Cas9 plasmid, the most efficient one, in three independent single cell cloning round. The potential importance of IRAK1 activity for EBNA1 function requires the analysis of more clones transfected with different IRAK1^{-} CRISPR/Cas 9 plasmids as well as further investigations.

In summary, our results demonstrate that IRAK4 is essential and very efficient to inhibit EBV lytic reactivation upon TLR9-induction. The TLR9-MyD88-IRAK4 pathway activation favors EBV latency by post-translational modifications and is largely protein synthesis independent. Therefore, the TLR9-MyD88-IRAK4 pathway is a potential therapeutic target to support disruption of EBV latency in EBV-associated lymphoproliferative disorders.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgement

This work was supported by grants from Oncosuisse and the Swiss National Foundation.

References

1. **Young LS**, **Rickinson AB**. 2004. Epstein-Barr virus: 40 years on. Nat Rev Cancer **4**:757–68.

2. **Speck SH**, **Ganem D**. 2010. Viral latency and its regulation: lessons from the gamma-herpesviruses. Cell Host Microbe **8**:100–15.

3. **Klein G**. 1994. Epstein-Barr virus strategy in normal and neoplastic B cells. Cell **77**:791–3.

4. **Brady G**, **Macarthur GJ**, **Farrell PJ**. 2008. Epstein-Barr virus and Burkitt lymphoma. Postgrad Med J **84**:372–7.

5. **Nemerow GR**, **Mold C**, **Schwend VK**, **Tollefson V**, **Cooper NR**. 1987. Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d. J Virol **61**:1416–20.

6. **Rowe DT**, **Rowe M**, **Evan GI**, **Wallace LE**, **Farrell PJ**, **Rickinson AB**. 1986. Restricted expression of EBV latent genes and T-lymphocyte-detected membrane antigen in Burkitt's lymphoma cells. EMBO J **5**:2599–607.

7. **Rowe M**, **Kelly GL**, **Bell AI**, **Rickinson AB**. 2009. Burkitt's lymphoma: the Rosetta Stone deciphering Epstein-Barr virus biology. Semin Cancer Biol **19**:377–88.

8. **Countryman J**, **Jenson H**, **Seibl R**, **Wolf H**, **Miller G**. 1987. Polymorphic proteins encoded within BZLF1 of defective and standard Epstein-Barr viruses disrupt latency. J Virol **61**:3672–9.

9. **Tovey MG**, **Lenoir G**, **Begon-Lours J**. 1978. Activation of latent Epstein-Barr virus by antibody to human IgM. Nature **276**:270–2.

10. **zur Hausen H**, **O'Neill FJ**, **Freese UK**, **Hecker E**. 1978. Persisting oncogenic herpesvirus induced by the tumour promotor TPA. Nature **272**:373–5.

11. **Luka J**, **Kallin B**, **Klein G**. 1979. Induction of the Epstein-Barr virus (EBV) cycle in latently infected cells by n-butyrate. Virology **94**:228–31.

12. **Takada K**. 1984. Cross-linking of cell surface immunoglobulins induces Epstein-Barr virus in Burkitt lymphoma lines. Int J Cancer **33**:27–32.

13. **di Renzo L**, **Altiok A**, **Klein G**, **Klein E**. 1994. Endogenous TGF-beta contributes to the induction of the EBV lytic cycle in two Burkitt lymphoma cell lines. Int J Cancer **57**:914–9.

14. **Lünemann A**, **Rowe M**, **Nadal D**. 2015. Epstein Barr Virus Volume 2: One Herpes Virus: Many Diseases, p. 265–287. *In* Münz, C (ed.), . Springer International Publishing, Cham.

15. **Middeldorp JM**. 2015. Epstein Barr Virus Volume 2: One Herpes Virus: Many Diseases, p. 289–323. *In* Münz, C (ed.), . Springer International Publishing, Cham.

16. **Hislop AD**, **Taylor GS**. 2015. Epstein Barr Virus Volume 2: One Herpes Virus: Many Diseases, p. 325–353. *In* Münz, C (ed.), . Springer International Publishing, Cham.

17. **Murata T**, **Kondo Y**, **Sugimoto A**, **Kawashima D**, **Saito S**, **Isomura H**, **Kanda T**, **Tsurumi T**. 2012. Epigenetic histone modification of Epstein-Barr virus BZLF1 promoter during latency and reactivation in Raji cells. J Virol **86**:4752–61.

18. **Ramasubramanyan S**, **Osborn K**, **Flower K**, **Sinclair AJ**. 2012. Dynamic chromatin environment of key lytic cycle regulatory regions of the Epstein-Barr virus genome. J Virol **86**:1809–19.

19. **Bryant H**, **Farrell PJ**. 2002. Signal Transduction and Transcription Factor Modification during Reactivation of Epstein-Barr Virus from Latency. J Virol **76**:10290–8.

20. **Ladell K**, **Dorner M**, **Zauner L**, **Berger C**, **Zucol F**, **Bernasconi M**, **Niggli FK**, **Speck RF**, **Nadal D**. 2007. Immune activation suppresses initiation of lytic Epstein-Barr virus infection. Cell Microbiol **9**:2055–69.

21. **Zauner L**, **Melroe GT**, **Sigrist J A**, **Rechsteiner MP**, **Dorner M**, **Arnold M**, **Berger**

570 **C**, **Bernasconi M**, **Schaefer BW**, **Speck RF**, **Nadal D**. 2010. TLR9 triggering in
571 Burkitt's lymphoma cell lines suppresses the EBV BZLF1 transcription via histone
572 modification. Oncogene **29**:4588–98.

573 22. **Kawai T**, **Akira S**. 2010. The role of pattern-recognition receptors in innate
574 immunity: update on Toll-like receptors. Nat Immunol **11**:373–84.

575 23. **Takeuchi O**, **Akira S**. 2010. Pattern recognition receptors and inflammation. Cell
576 **140**:805–20.

577 24. **Heeg K**, **Dalpke A**, **Peter M**, **Zimmermann S**. 2008. Structural requirements for
578 uptake and recognition of CpG oligonucleotides. Int J Med Microbiol **298**:33–8.

579 25. **Takeshita F**, **Gursel I**, **Ishii KJ**, **Suzuki K**, **Gursel M**, **Klinman DM**. 2004. Signal
580 transduction pathways mediated by the interaction of CpG DNA with Toll-like
581 receptor 9. Semin Immunol **16**:17–22.

582 26. **Rechsteiner MP**, **Berger C**, **Zauner L**, **Sigrist J A**, **Weber M**, **Longnecker R**,
583 **Bernasconi M**, **Nadal D**. 2008. Latent membrane protein 2B regulates
584 susceptibility to induction of lytic Epstein-Barr virus infection. J Virol **82**:1739–47.

585 27. **Coban C**, **Ishii KJ**, **Kawai T**, **Hemmi H**, **Sato S**, **Uematsu S**, **Yamamoto M**,
586 **Takeuchi O**, **Itagaki S**, **Kumar N**, **Horii T**, **Akira S**. 2005. Toll-like receptor 9
587 mediates innate immune activation by the malaria pigment hemozoin. J Exp Med
588 **201**:19–25.

589 28. **Aya T**, **Kinoshita T**, **Imai S**, **Koizumi S**, **Mizuno F**, **Osato T**, **Satoh C**, **Oikawa T**,
590 **Kuzumaki N**, **Ohigashi H**. 1991. Chromosome translocation and c-MYC activation
591 by Epstein-Barr virus and Euphorbia tirucalli in B lymphocytes. Lancet (London,
592 England) **337**:1190.

593 29. **Flemington EK**, **Goldfeld AE**, **Speck SH**. 1991. Efficient transcription of the

Epstein-Barr virus immediate-early BZLF1 and BRLF1 genes requires protein synthesis. J Virol **65**:7073–7.

30. **Yin Q**, **Jupiter K**, **Flemington EK**. 2004. The Epstein-Barr virus transactivator Zta binds to its own promoter and is required for full promoter activity during anti-Ig and TGF-beta1 mediated reactivation. Virology **327**:134–43.

31. **Ye J**, **Gradoville L**, **Miller G**. 2010. Cellular immediate-early gene expression occurs kinetically upstream of Epstein-Barr virus bzlf1 and brlf1 following cross-linking of the B cell antigen receptor in the Akata Burkitt lymphoma cell line. J Virol **84**:12405–18.

32. **Ran FA**, **Hsu PD**, **Wright J**, **Agarwala V**, **Scott DA**, **Zhang F**. 2013. Genome engineering using the CRISPR-Cas9 system. Nat Protoc **8**:2281–308.

33. **Picard C**, **Puel A**, **Bonnet M**, **Ku C**, **Bustamante J**, **Yang K**, **Soudais C**, **Dupuis S**, **Feinberg J**, **Fieschi C**, **Elbim C**, **Hitchcock R**, **Lammas D**, **Davies G**, **Al-Ghonaium A**, **Al-Rayes H**, **Al-Jumaah S**, **Al-Hajjar S**, **Al-Mohsen IZ**, **Frayha HH**, **Rucker R**, **Hawn TR**, **Aderem A**, **Tufenkeji H**, **Haraguchi S**, **Day NK**, **Good RA**, **Gougerot-Pocidalo M-A**, **Ozinsky A**, **Casanova J-L**. 2003. Pyogenic bacterial infections in humans with IRAK-4 deficiency. Science **299**:2076–9.

34. **Lye E**, **Mirtsos C**, **Suzuki N**, **Suzuki S**, **Yeh W-C**. 2004. The role of interleukin 1 receptor-associated kinase-4 (IRAK-4) kinase activity in IRAK-4-mediated signaling. J Biol Chem **279**:40653–8.

35. **Motshwene PG**, **Moncrieffe MC**, **Grossmann JG**, **Kao C**, **Ayaluru M**, **Sandercock AM**, **Robinson C V**, **Latz E**, **Gay NJ**. 2009. An oligomeric signaling platform formed by the Toll-like receptor signal transducers MyD88 and IRAK-4. J Biol Chem **284**:25404–11.

36. **Jenkins PJ**, **Binné UK**, **Farrell PJ**. 2000. Histone acetylation and reactivation of Epstein-Barr virus from latency. J Virol **74**:710–20.

37. **Countryman JK**, **Gradoville L**, **Miller G**. 2008. Histone hyperacetylation occurs on promoters of lytic cycle regulatory genes in Epstein-Barr virus-infected cell lines which are refractory to disruption of latency by histone deacetylase inhibitors. J Virol **82**:4706–19.

38. **Henault M**, **Lee LN**, **Evans GF**, **Zuckerman SH**. 2005. The human Burkitt lymphoma cell line Namalwa represents a homogenous cell system characterized by high levels of Toll-like receptor 9 and activation by CpG oligonucleotides. J Immunol Methods **300**:93–9.

39. **Saraiva M**, **O'Garra A**. 2010. The regulation of IL-10 production by immune cells. Nat Rev Immunol **10**:170–81.

40. **Kalampokis I**, **Yoshizaki A**, **Tedder TF**. 2013. IL-10-producing regulatory B cells (B10 cells) in autoimmune disease. Arthritis Res Ther **15 Suppl 1**:S1.

41. **Haas F**, **Yamauchi K**, **Murat M**, **Bernasconi M**, **Yamanaka N**, **Speck RF**, **Nadal D**. 2014. Activation of NF-κB via endosomal Toll-like receptor 7 (TLR7) or TLR9 suppresses murine herpesvirus 68 reactivation. J Virol **88**:10002–12.

42. **Izumiya Y**, **Izumiya C**, **Hsia D**, **Ellison TJ**, **Luciw P a**, **Kung H-J**. 2009. NF-kappaB serves as a cellular sensor of Kaposi's sarcoma-associated herpesvirus latency and negatively regulates K-Rta by antagonizing the RBP-Jkappa coactivator. J Virol **83**:4435–46.

43. **Callan MFC**. 2004. The immune response to Epstein-Barr virus. Microbes Infect **6**:937–45.

44. **Duellman SJ**, **Thompson KL**, **Coon JJ**, **Burgess RR**. 2009. Phosphorylation sites of

Epstein-Barr virus EBNA1 regulate its function. J Gen Virol **90**:2251–9.

Figure Legends

Figure 1: Experimental design for the establishment of CRISPR/Cas9 single cell clones. Steps for reagent design, construction, cell line expansion and characterization are depicted. Three different custom sgRNAs (light blue bars) were designed *in silico* via the CRISPR Design Tool (http://crispr.mit.edu/). sgRNA guide sequences were cloned into the expression plasmid pSpCas9(BB)-2A-GFP (PX458) bearing sgRNA scaffold backbone (BB), Cas9, and GFP. Cloned and sequence-verified pSpCas9(BB)-2A-GFP (PX458) plasmids were then electroporated into Akata cells, after 48h, GFP-positive cells were single cell sorted by FACS. Finally, the GFP positive single cell clones were expanded, genotyped and characterized. (Adapted from (32))

Figure 2: TLR9 triggering via ODN CpG 2006 inhibits EBV master lytic gene *BZLF1* mRNA expression in BL cells despite protein synthesis inhibition with cycloheximide and 4E1RCat.

(**a**) Akata cells were treated with the protein synthesis inhibitors cycloheximide (33 μg/ml) or 4E1RCat (10 μM), for 30 min, 2h or 8h or with their vehicles, H_2O or DMSO, respectively, for 8h. Cell viability was determined by Trypan blue exclusion assay. Untreated and vehicle-treated cells (H_2O for cycloheximide and DMSO for 4E1RCat) were used as negative controls; digitonin (30 μg/ml) treated cells as a positive control for cell toxicity after 8.5h incubation. Results are shown as percentage of viable cells (n=2). (**b**) and (**c**) Protein synthesis inhibitors cycloheximide (33 μg/ml) and 4E1RCat (10 μM), were added first to the Akata cells. After 30 min incubation, cells were treated for 2h with ODN CpG 2006, and finally for 6h with anti-IgG. After a total incubation time of 8.5h the cells were lysed and the mRNA extracted. The *BZLF1* mRNA level relative to

HMBS was measured by RT-qPCR. (n=3). (**b**) Represents an enlargement of the *BZLF1* mRNA levels measured upon incubation with ODN CpG 2006, cycloheximide or 4E1RCat only, which is presented in (**c**). Data are represented as mean ± SD. Statistics were calculated using the unpaired *t* test. (*, $P<0.05$)

Figure 3: CRISPR/Cas9 mutation of TLR9, MyD88, IRAK4 and IRAK1 result in a complete TLR9 signaling abrogation, and affect EBV viral copy numbers per cells. Akata Burkitt's lymphoma B cells transfected with px458 plasmids coding for Cas9 and containing a sgRNA targeting TLR9 (TLR9^{-}b3, TLR9^{-}b5 and TLR9^{-}b6), MyD88 (MyD88^{-}a3, MyD88^{-}a5 and MyD88^{-}b1), IRAK4 (IRAK4^{-}a3 and IRAK4^{-}a4) and IRAK1 (IRAK1^{-}b2 and IRAK1^{-}b8), respectively, were diluted for single cell cloning, sequenced and clones containing an early stop codon were selected for further characterization. (**a**) % of IgG positive cells was measured by flow cytometry. (**b**) *TLR9* mRNA level was measured by RT-qPCR and normalized over *HMBS* and over WT Akata cells. (**c**) IL-10 cytokine expression level measured by ELISA in the supernatant of untreated cells, and cells treated for 8h ODN CpG 2006. (**d**) Viral *BamH1 W* copy numbers over cellular *HMBS* determined by qPCR. (**a**) Has been performed only once (**b, c, d**) Shows one representative experiment out of three. Data are represented as mean ± SD

Figure 4: TLR9, MyD88 or IRAK4 inactivation inhibits NF-κB nuclear translocation upon TLR9 activation with ODN CpG 2006.
Results show NF-κB p65 protein expression in the cytoplasmic and nuclear fraction of Akata WT cells and TLR9 (TLR9^{-}b3, TLR9^{-}b5 and TLR9^{-}b6), MyD88 (MyD88^{-}a3, MyD88^{-}a5 and MyD88^{-}b1) and IRAK4 (IRAK4^{-}a3 and IRAK4^{-}a4) CRISPR single cell clones. Cells were treated with ODN CpG 2006 for 90'. Lamin A/C and tubulin protein expressions

were measured as loading control for the cytoplasmic respectively nuclear fractions. One representative experiment is shown out of three independent experiments.

Figure 5: TLR9-induced inhibition of *BZLF1* expression is strictly TLR9, MyD88 and IRAK4 dependent.
CRISPR/Cas9 mutated TLR9, MyD88 and IRAK4 clones were treated for 2h with ODN CpG 2006 prior to lytic reactivation by Ig-crosslinking with anti-IgG for 6h. *BZLF1* mRNA expression level normalized to *HMBS* was measured by RT-qPCR and relative over untreated cells in TLR9 clones, MyD88 clones and IRAK4 clones. Shown is one representative experiment out of three. Data are represented as mean ± SD (n=2). Statistics were calculated using the unpaired *t* test. (***, $P<0.001$; *, $P<0.05$; n.s., not significant).

Table 1: CRISPR single guide RNA target sequences.

Target name	Genomic locus	Target Sequence (5'-3')
IRAK4$^-$ a	IRAK4 exon 2	AGGCAGCGCACATATGTTGATGG
IRAK4$^-$ b	IRAK4 exon 2	TATGTGCGCTGCCTCAATGTTGG
IRAK4$^-$ c	IRAK4 exon 2	GCCTCAATGTTGGACTAATTAGG
IRAK1$^-$ a	IRAK1 exon 2	CGGTCTGGTCGCGCACGATCAGG
IRAK1$^-$ b	IRAK1 exon 2	GATCAACCGCAACGCCCGTGTGG
IRAK1$^-$ c	IRAK1 exon 2	GGTCTGGTCGCGCACGATCAGGG
MyD88$^-$ a	MyD88 exon 1	GTTCTTGAACGTGCGGACACAGG
MyD88$^-$ b	MyD88 exon 1	GCTCCAGCAGCACGTCGTCGCGG
MyD88$^-$ c	MyD88 exon 3	ATGAAGGCATCGAAACGCTCAGG
TLR9$^-$ a	TLR9 exon 2	CGCTGATGCGGTTGTCCGACAGG
TLR9$^-$ b	TLR9 exon 2	ACTGGGTGTACAACGAGCTTCGG
TLR9$^-$ c	TLR9 exon 2	GCTCACGGCTATTCGGCCGTGGG

Table 2: Sequencing summary of CRISPR/Cas9 IRAK4$^-$, TLR9$^-$ and MyD88$^-$ clones.

Clones	DNA sequence changes	Amino acid change
IRAK4$^-$ a3	c.25inA	p.Thr9AspfsX19
IRAK4$^-$ a4	c.24delA	p.Thr9HisX17

TLR9$^-$ b3	c.2615_2690delCCTTCGTGGTCTTCGACAAAACGCAGAGC GCAGTGGCAGACTGGGTGTACAACGAGCTTCGGGGGCA GCTGGAGGA + c.2692T>C	p.Ala872GlyX945
TLR9$^-$ b5	c.2672inC	p.Leu891ProfsX921
TLR9$^-$ b6 1*	c.2672inC	p.Leu891ProfsX921
TLR9$^-$ b6 2*	c.2655_2682delCTGGGTGTACAACGAGCTTCGGGGGCAG	p.Val887ArgfsX960
MyD88$^-$ a3	c.153_165delGTGCGGACACAG	p.Arg53AlafsX122
MyD88$^-$ a5	c.161delC	p.Thr54AspfsX99
MyD88$^-$ b1	c.336delC	p.Asp112GlufsX273

708 DNA sequence changes nomenclature is based on the coding DNA sequence. * TLR9$^-$ b6
709 derives from two different clones whose sequence was determined by TA cloning and
710 sequencing.
711
712 **Table 3**: Summary of the CRISPR Cas9 transfection and characterization experiments.

	IRAK1$^-$	**IRAK4$^-$**	**TLR9$^-$**	**MyD88$^-$**	**Akata px458-ctl**
Inactive TLR9 pathway (ko)	13/23[a]	4/10[a]	5/10[a]	6/10[a]	0/12[a]
EBV positive	7/23[b]	6/10[b]	6/10[b]	9/10[b]	10/12[b]
EBV positive / TLR9 ko	0/13[c]	3/4[c]	3/5[c]	5/6[c]	0/0[c]

714
715
716
717

718 [a] = number of single cell clones with an inactive TLR9 pathway over the total number of
719 single cell clones analyzed; [b] = number of EBV positive single cell clones over the total
720 number of single cell clones analyzed; [c] = number of EBV positive single cell clones over
721 the number of single cell clones with an inactive TLR9 pathway.

Figure 1:

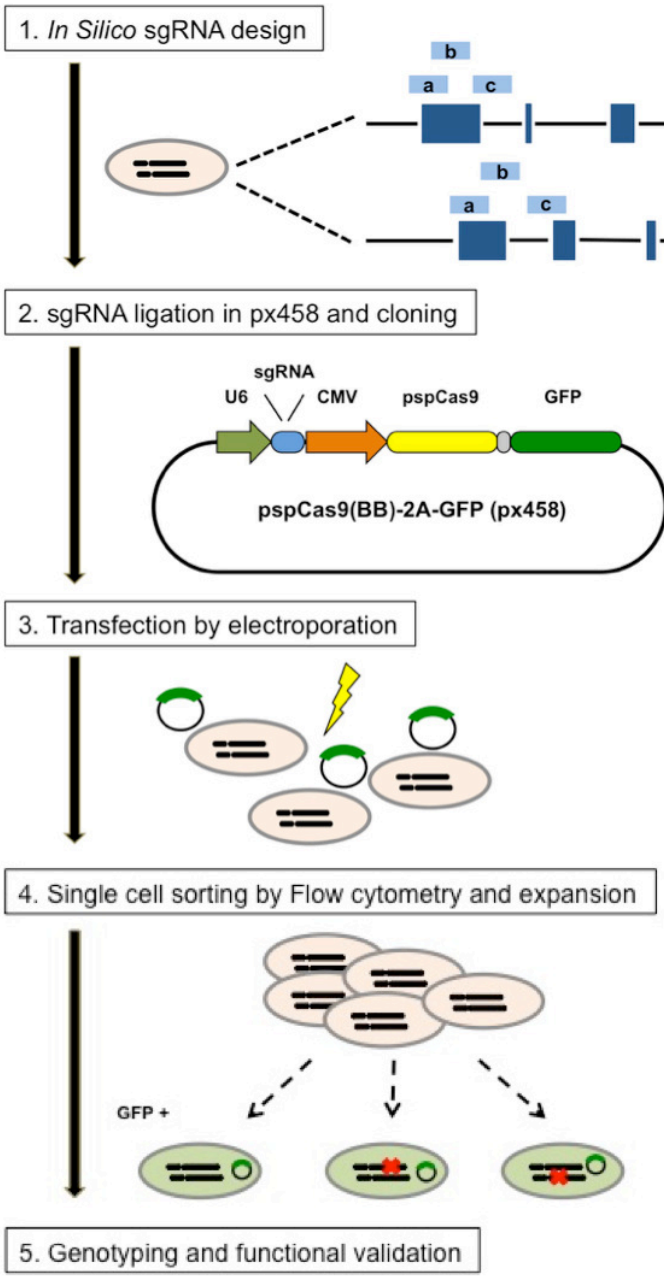

1. *In Silico* sgRNA design
b
a
c
b
a
c
2. sgRNA ligation in px458 and cloning
sgRNA
U6
CMV
pspCas9
GFP
pspCas9(BB)-2A-GFP (px458)
3. Transfection by electroporation
4. Single cell sorting by Flow cytometry and expansion
GFP +
5. Genotyping and functional validation

Figure 2:

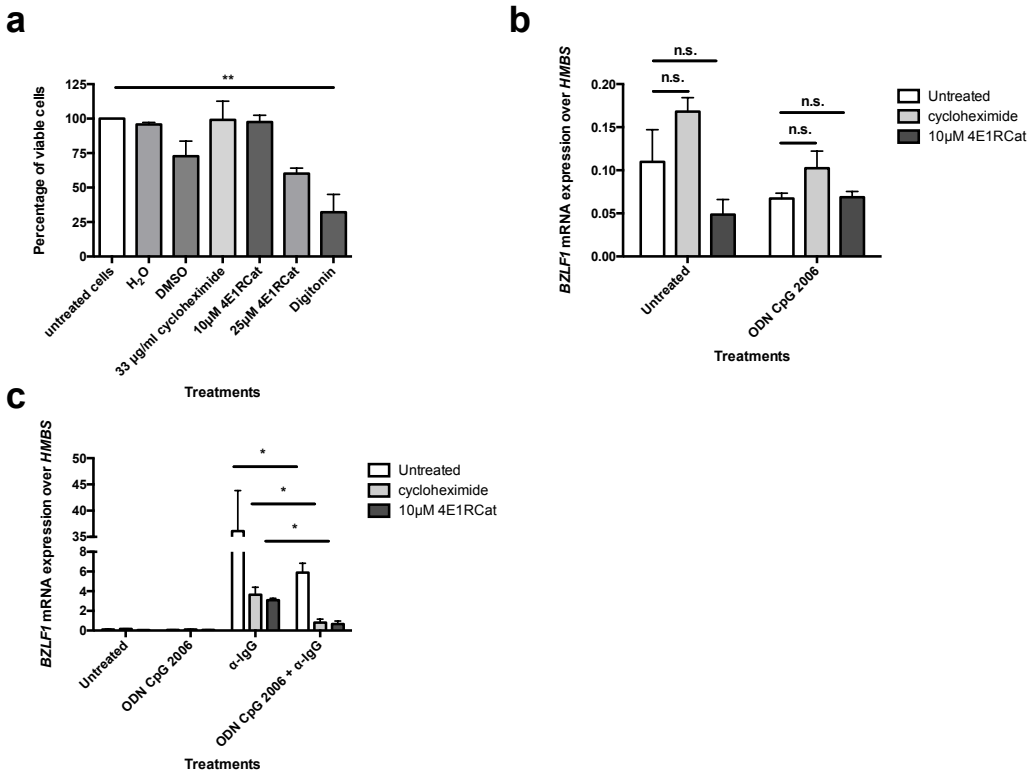

a
Percentage of viable cells
125
100
75
50
25
0
**
untreated cells
H2O
DMSO
33 µg/ml cycloheximide
10µM 4E1RCat
25µM 4E1RCat
Digitonin
Treatments
b
BZLF1 mRNA expression over HMBS
0.20
0.15
0.10
0.05
0.00
n.s.
n.s.
n.s.
n.s.
Untreated
ODN CpG 2006
Treatments
Untreated
cycloheximide
10µM 4E1RCat
c
BZLF1 mRNA expression over HMBS
50
45
40
35
8
6
4
2
0
*
*
*
Untreated
ODN CpG 2006
c-IgG
ODN CpG 2006 + c-IgG
Treatments
Untreated
cycloheximide
10µM 4E1RCat

Figure 3:

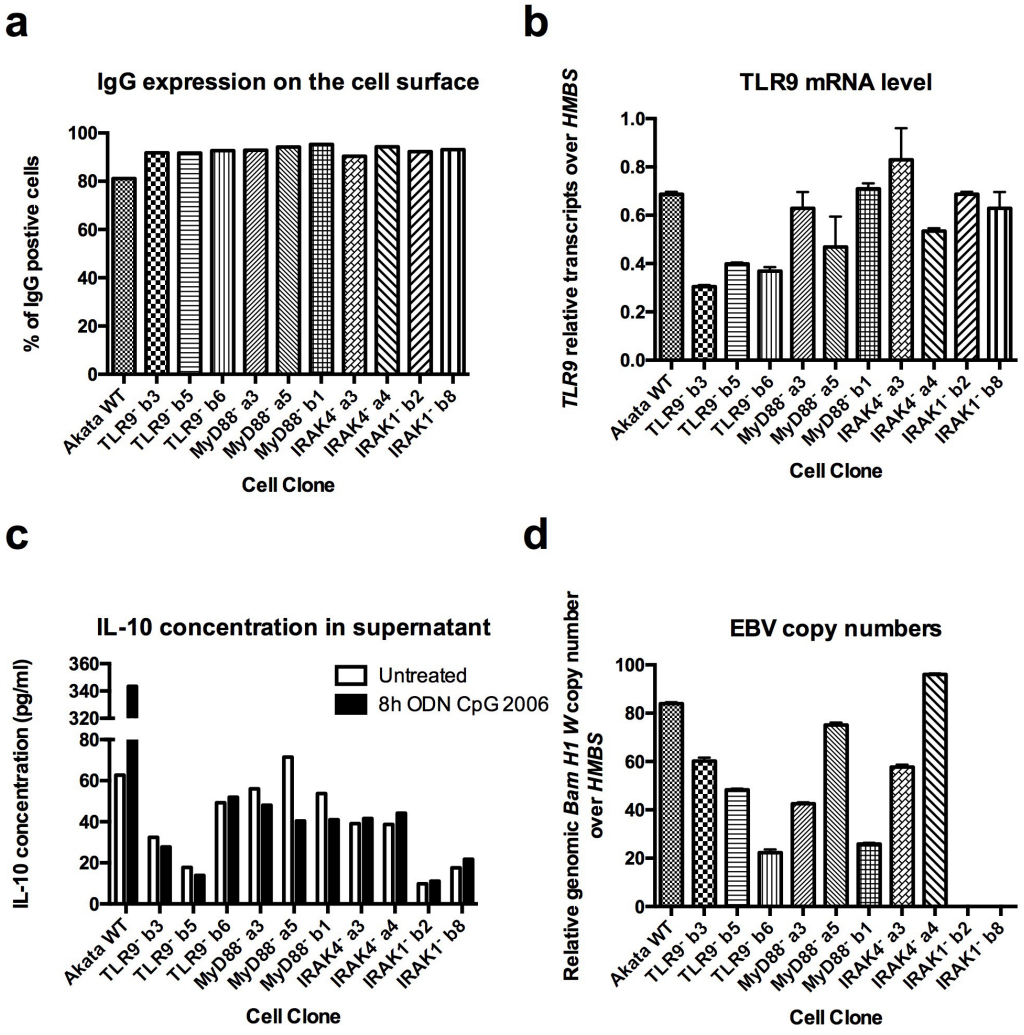

a
IgG expression on the cell surface
% of IgG postive cells
100
80
60
40
20
0
Akata WT
TLR9- b3
TLR9- b5
TLR9- b6
MyD88- a3
MyD88- a5
MyD88- b1
IRAK4- a3
IRAK4- a4
IRAK1- b2
IRAK1- b8
Cell Clone
b
TLR9 mRNA level
TLR9 relative transcripts over HMBS
1.0
0.8
0.6
0.4
0.2
0.0
Cell Clone
c
IL-10 concentration in supernatant
IL-10 concentration (pg/ml)
Untreated
8h ODN CpG 2006
360
340
320
80
60
40
20
0
Cell Clone
d
EBV copy numbers
Relative genomic Bam H1 W copy number over HMBS
100
80
60
40
20
0
Cell Clone

Figure 4:

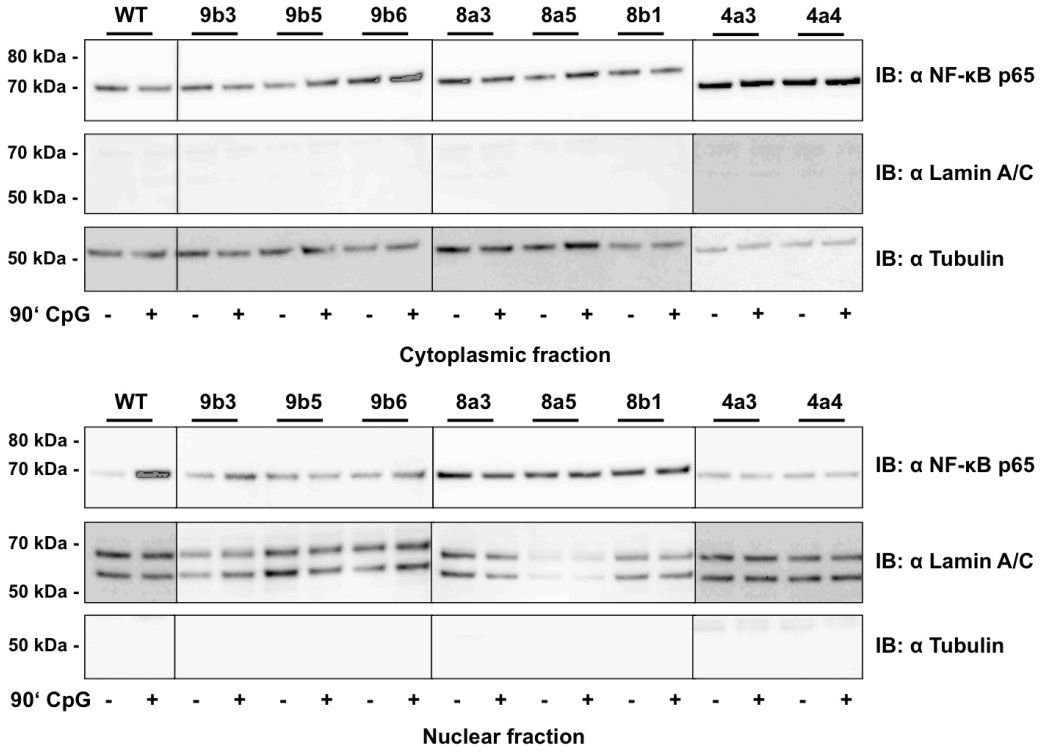
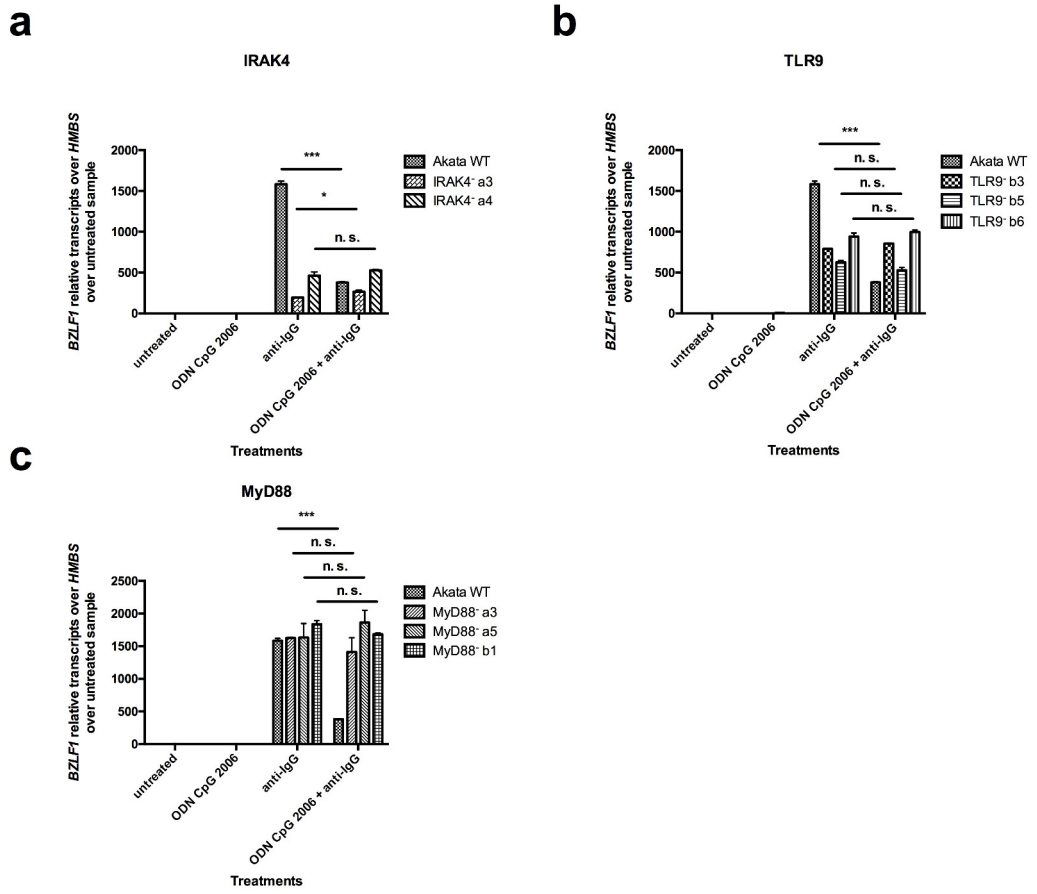
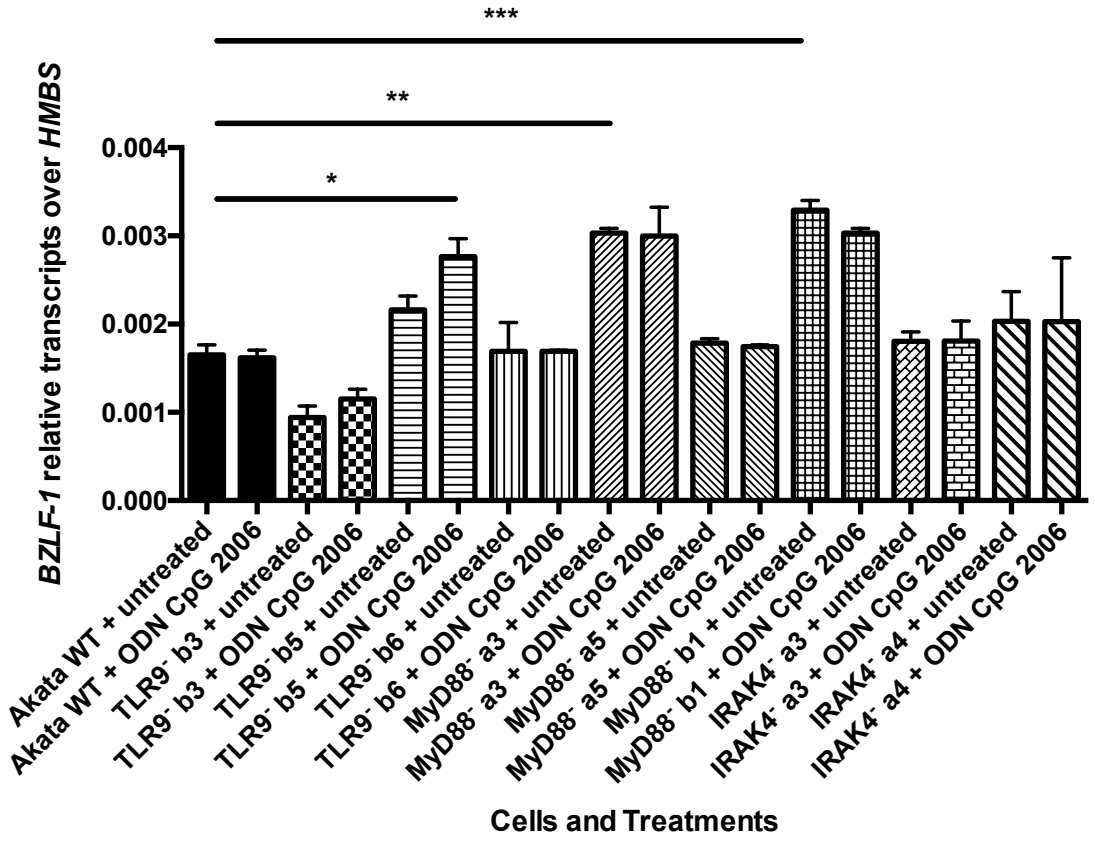

WT
9b3
9b5
9b6
8a3
8a5
8b1
4a3
4a4
80 kDa -
70 kDa -
IB: α NF-κB p65
70 kDa -
50 kDa -
IB: α Lamin A/C
50 kDa -
IB: α Tubulin
90‘ CpG - + - + - + - + - + - + - + - + - +
Cytoplasmic fraction
WT
9b3
9b5
9b6
8a3
8a5
8b1
4a3
4a4
80 kDa -
70 kDa -
IB: α NF-κB p65
70 kDa -
50 kDa -
IB: α Lamin A/C
50 kDa -
IB: α Tubulin
90‘ CpG - + - + - + - + - + - + - + - + - +
Nuclear fraction

Figure 5:


a
IRAK4
BZLF1 relative transcripts over HMBS over untreated sample
2000
1500
1000
500
0
***
*
n. s.
Akata WT
IRAK4- a3
IRAK4- a4
untreated
ODN CpG 2006
anti-IgG
ODN CpG 2006 + anti-IgG
Treatments
b
TLR9
BZLF1 relative transcripts over HMBS over untreated sample
2000
1500
1000
500
0
***
n. s.
n. s.
n. s.
Akata WT
TLR9- b3
TLR9- b5
TLR9- b6
untreated
ODN CpG 2006
anti-IgG
ODN CpG 2006 + anti-IgG
Treatments
c
MyD88
BZLF1 relative transcripts over HMBS over untreated sample
2500
2000
1500
1000
500
0
***
n. s.
n. s.
n. s.
Akata WT
MyD88- a3
MyD88- a5
MyD88- b1
untreated
ODN CpG 2006
anti-IgG
ODN CpG 2006 + anti-IgG
Treatments

Supplementary Figure S1:

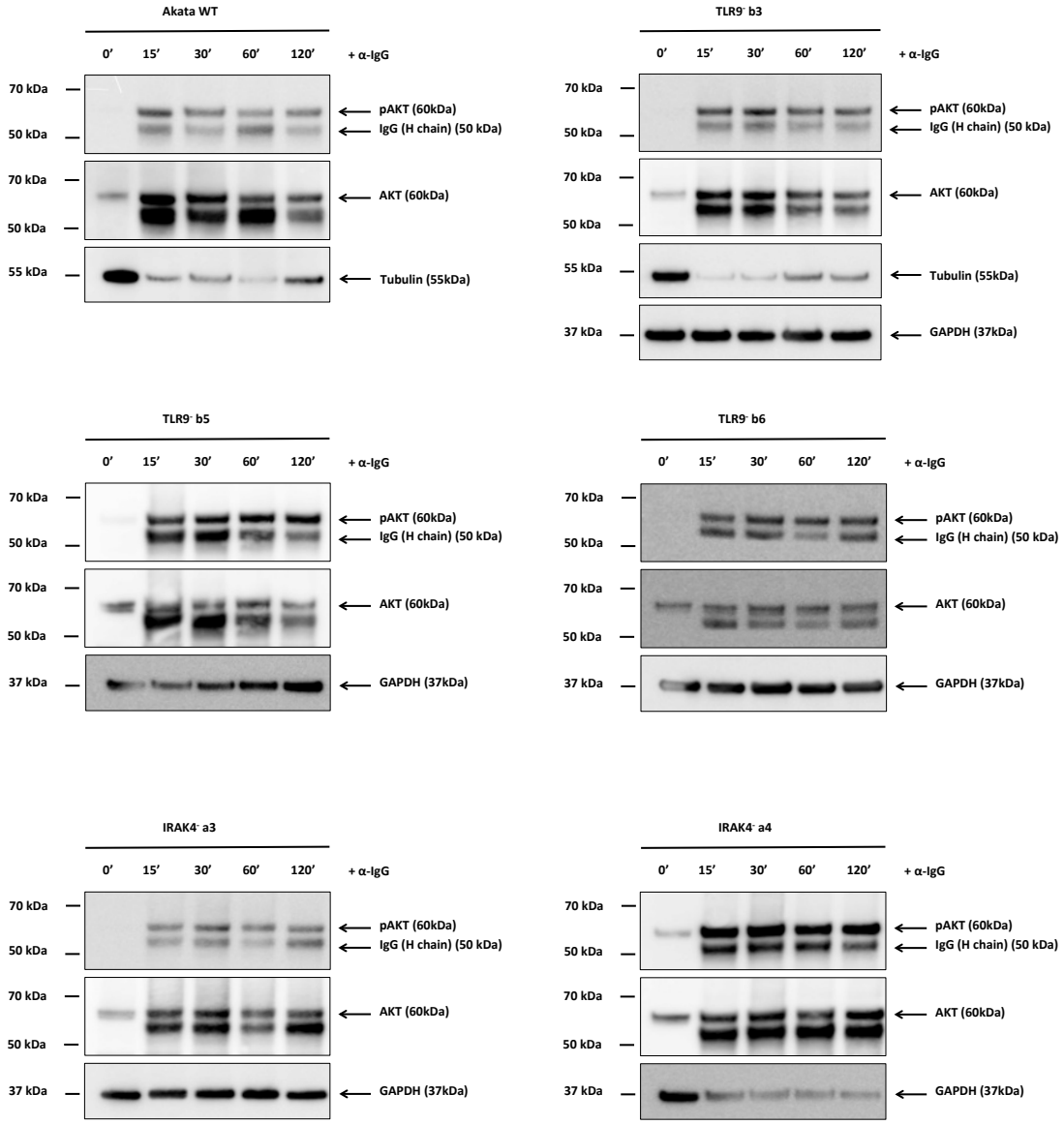
CRISPR/Cas9 mutated TLR9, MyD88 and IRAK4 clones were treated for 2h with ODN CpG 2006. *BZLF1* mRNA expression level normalized to *HMBS* was measured by RT-qPCR in TLR9$^-$ clones, MyD88$^-$ clones and IRAK4$^-$ clones. Shown is one representative experiment out of three. Data are represented as mean ± SD (n=2). Statistics were calculated using the unpaired *t* test. (***, $P<0.001$; *, $P<0.05$; n.s., not significant)**.**



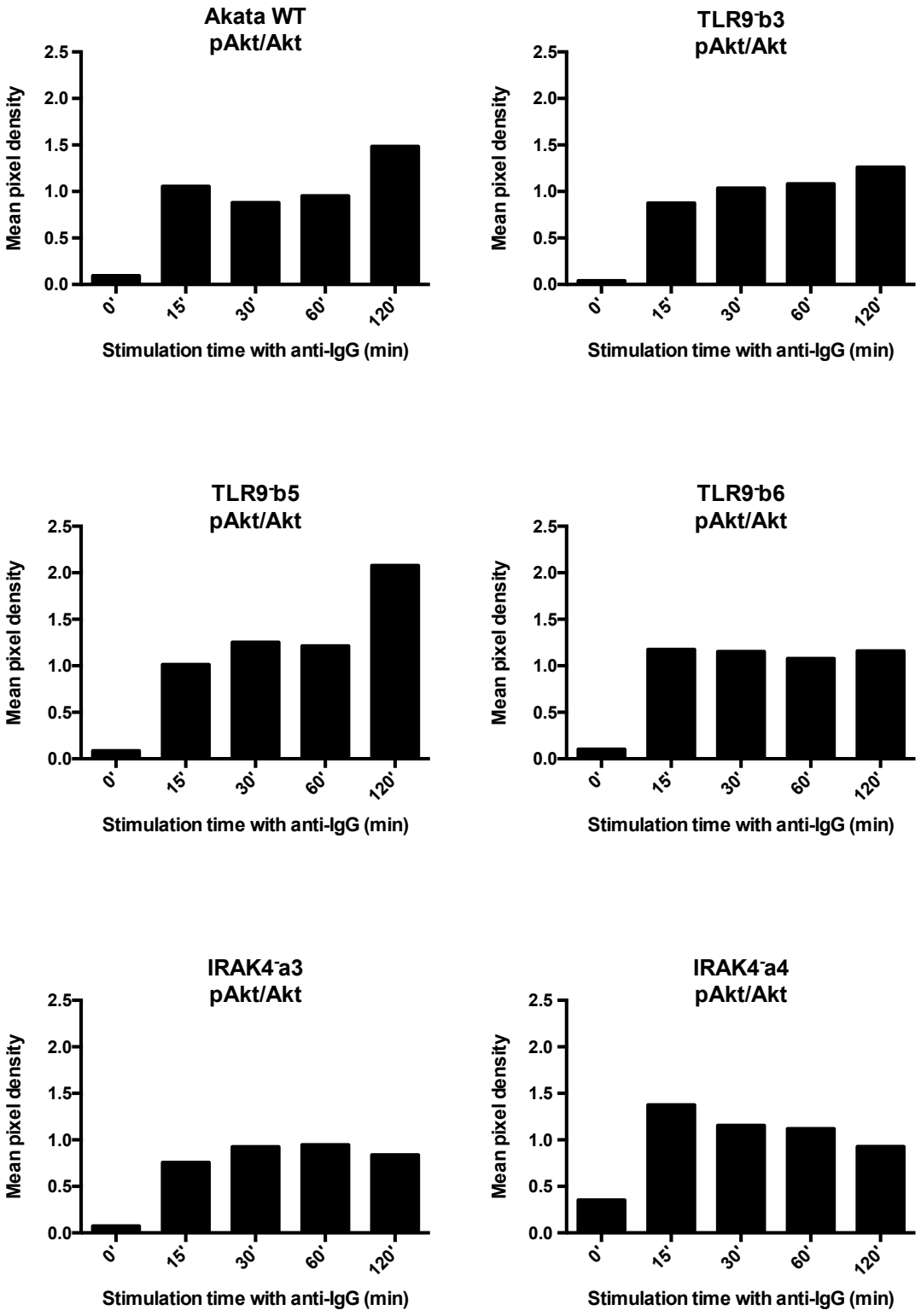
Supplementary Figure S2:

Akata WT cells and TLR9 (TLR9^{-}b3, TLR9^{-}b5 and TLR9^{-}b6) and IRAK4 (IRAK4^{-}a3 and IRAK4^{-}a4) CRISPR/Cas9 single cell clones were treated with ODN CpG 2006 for 15', 30', 60' and 120'. (**a**) Results show pAkt and Akt protein expression. GAPDH or Tubulin protein expressions were measured as loading control. (**b**) Graphical representation of the densitometric measurement of (**a**). pAkt values were calculated relatively to the Akt level. One representative experiment is shown.

a



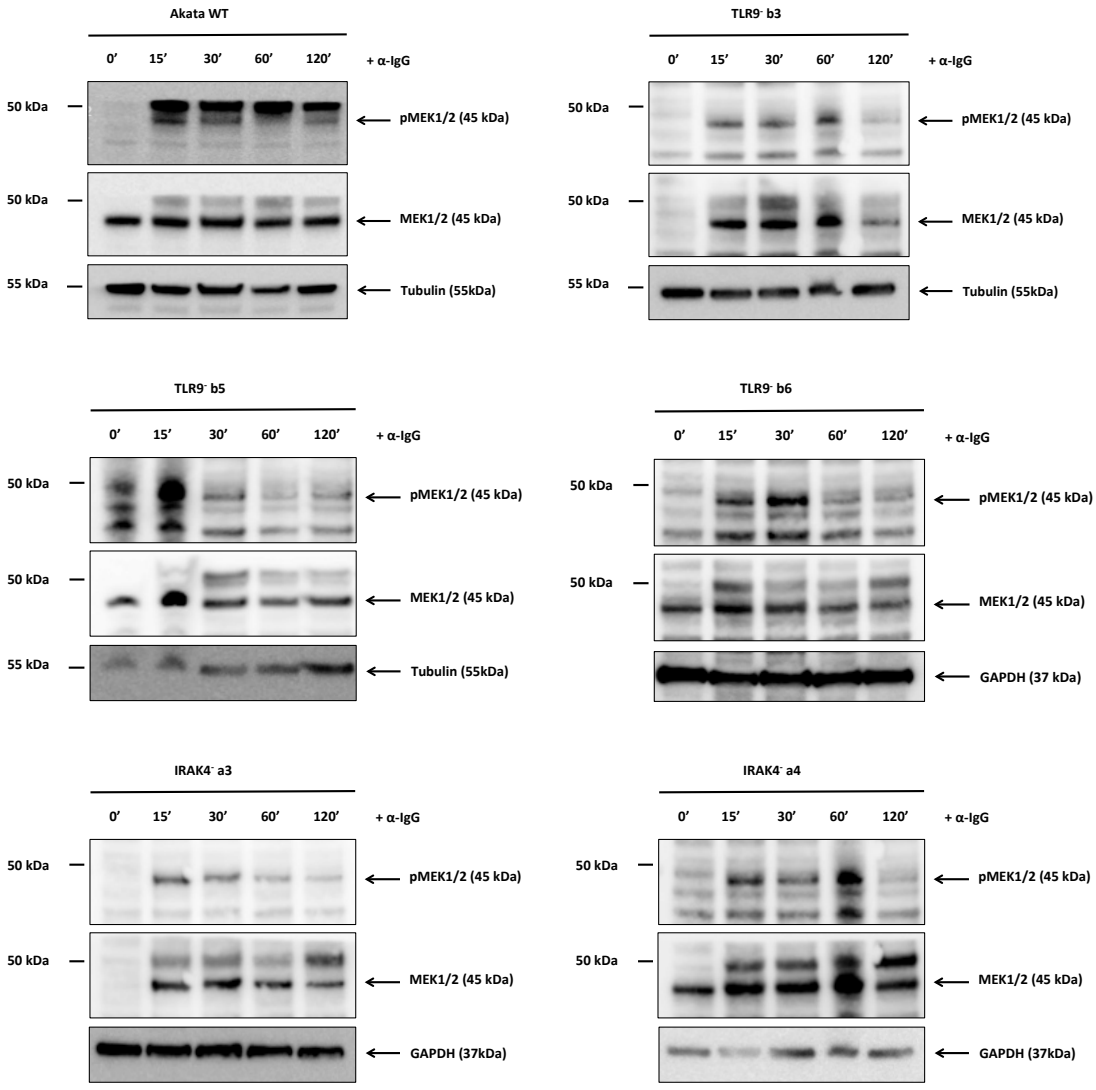
b


Akata WT
pAkt/Akt
TLR9-b3
pAkt/Akt
TLR9-b5
pAkt/Akt
TLR9-b6
pAkt/Akt
IRAK4-a3
pAkt/Akt
IRAK4-a4
pAkt/Akt
Mean pixel density
0.0
0.5
1.0
1.5
2.0
2.5
0'
15'
30'
60'
120'
Stimulation time with anti-IgG (min)

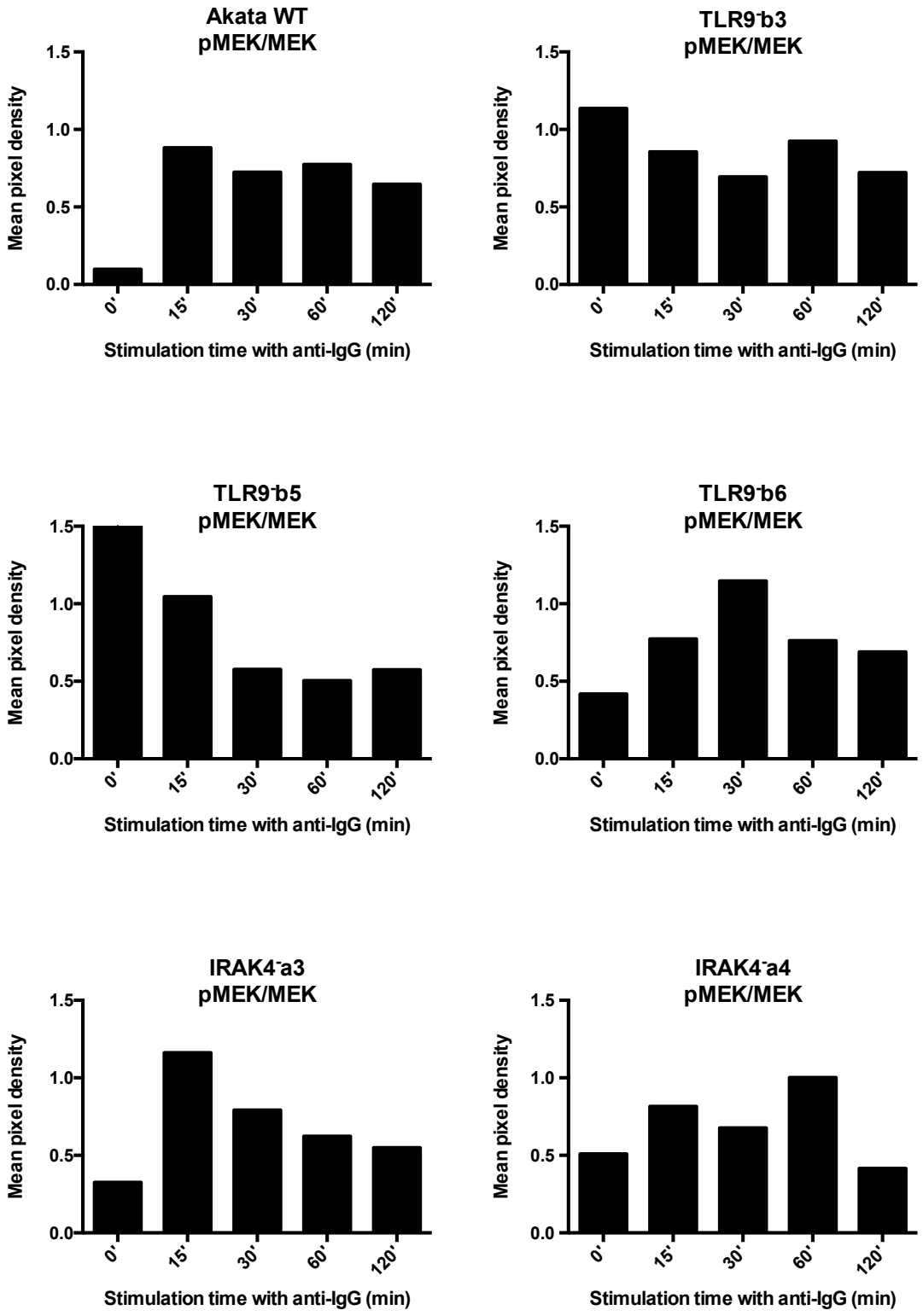
Supplementary Figure S3:

Akata WT cells and TLR9 (TLR9-b3, TLR9-b5 and TLR9-b6) and IRAK4 (IRAK4-a3 and IRAK4-a4) CRISPR/Cas9 single cell clones were treated with ODN CpG 2006 for 15', 30', 60' and 120'. (**a**) Results show pMek1/2 and Mek1/2 protein expression. GAPDH or Tubulin protein expressions were measured as loading control. (**b**) Graphical representation of the densitometric measurement of (**a**). pMek1/2 values were calculated relatively to the Mek1/2 level. One representative experiment is shown.

a



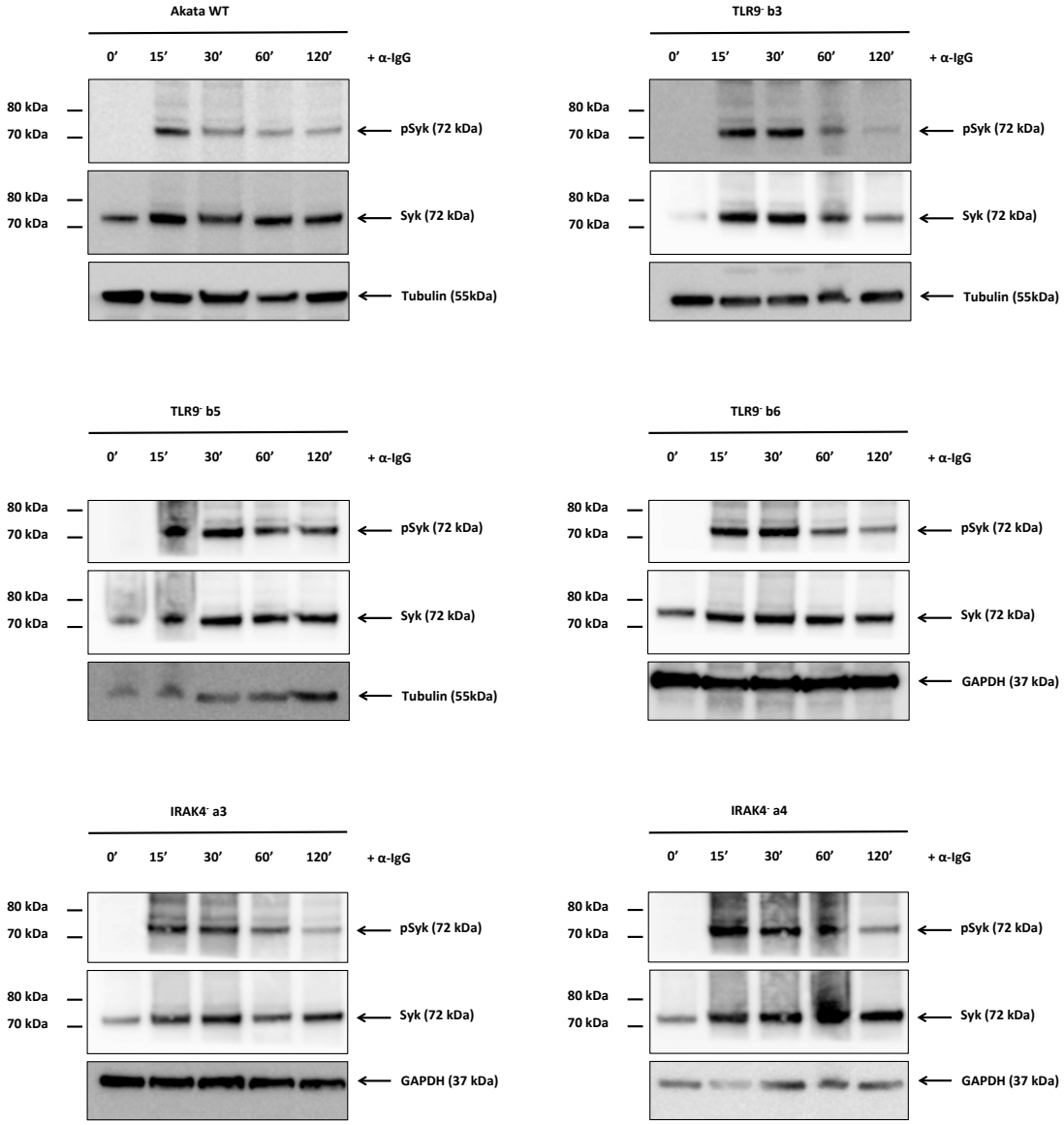
b


Akata WT
pMEK/MEK
TLR9-b3
pMEK/MEK
TLR9-b5
pMEK/MEK
TLR9-b6
pMEK/MEK
IRAK4-a3
pMEK/MEK
IRAK4-a4
pMEK/MEK
Mean pixel density
1.5
1.0
0.5
0.0
0'
15'
30'
60'
120'
Stimulation time with anti-IgG (min)

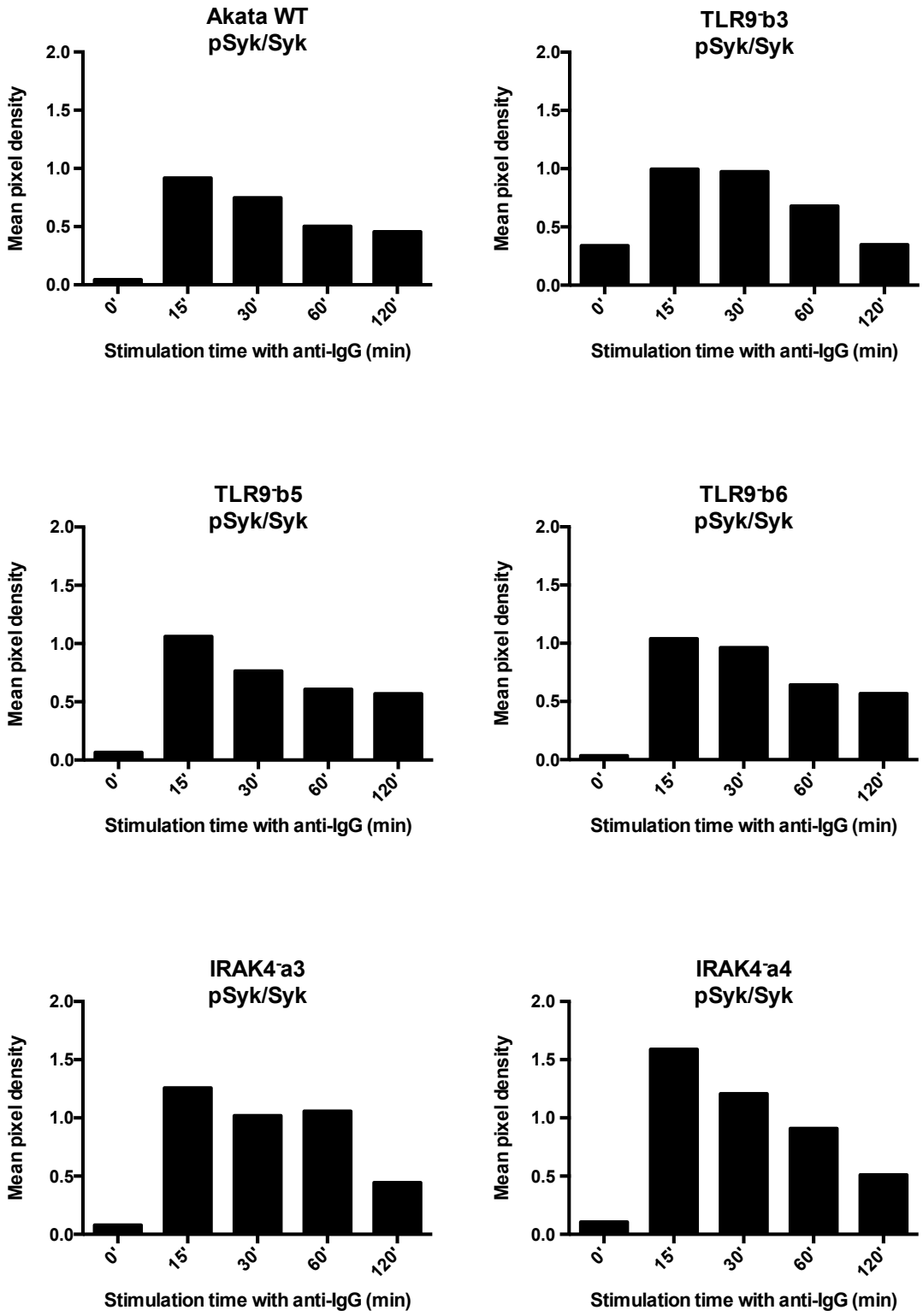
Supplementary Figure S4:

Akata WT cells and TLR9 (TLR9-b3, TLR9-b5 and TLR9-b6) and IRAK4 (IRAK4-a3 and IRAK4-a4) CRISPR/Cas9 single cell clones were treated with ODN CpG 2006 for 15', 30', 60' and 120'. (**a**) Results show pSyk and Syk protein expression. GAPDH or Tubulin protein expressions were measured as loading control. (**b**) Graphical representation of the densitometric measurement of (**a**). pSyk values were calculated relatively to the Syk level. One representative experiment is shown.

a



b


Akata WT
pSyk/Syk
TLR9-b3
pSyk/Syk
TLR9-b5
pSyk/Syk
TLR9-b6
pSyk/Syk
IRAK4-a3
pSyk/Syk
IRAK4-a4
pSyk/Syk
Mean pixel density
2.0
1.5
1.0
0.5
0.0
0'
15'
30'
60'
120'
Stimulation time with anti-IgG (min)